Taxonomy and Evolution in *Bupleurum* L. (*Umbelliferae*) in the W Mediterranean and Macaronesia

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Bupleurum fruticosum L.
Flowers

Bupleurum fruticosum L.

Jardim Botânico
Universidade de Coimbra

Bupleurum ranunculoides L.

Royal Botanic Garden Edinburgh

To my Parents
and Fátima Sales

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Contents

Abstract

Bupleurum L. (*Umbelliferae – Apioideae*) is a genus of c. 150 species, with a broad distribution in the N Hemisphere (except the S African *B. mundii* Cham. & Schltdl.), but with many species restricted to small areas. The most peculiar features of *Bupleurum*, almost unique in the family, are its simple, entire leaves which are parallel-veined in the vast majority of the species. The genus also contains several woody species, which are rare in umbellifers. The highest diversity in *Bupleurum*, morphological and species/taxa number, is found in the Mediterranean region. Western Mediterranean taxa, especially from NW Africa, required detailed revision as most of the endemics were very poorly known. The genus is generally regarded as a natural (monophyletic) and primitive (basal) group, but relationships among species, and its infrageneric classification needed much further research.

A revision of the taxonomy and nomenclature of the c. 30 species in the W Mediterranean (Iberian Peninsula, Balearic Islands & NW Africa) and Macaronesia (Canary Islands and Madeira) is presented. A key to identification of species, typification details (most species typified for the first time), morphological description, ecological data, detailed geographical distribution (with maps) and conservation status (if endangered) are included, together with critical notes on taxonomy and nomenclature; no new species are described. Morphological investigation was based on a large collection of herbarium material, and also, for some species, on living plants studied in the wild or during cultivation. Anatomical and SEM studies were also carried out to evaluate the usefulness of these techniques in the search of new taxonomic characters in the genus.

Phylogenetic relationships within the genus were investigated using the ITS region (internal transcribed spacers) of 18-26S nuclear ribosomal DNA repeat. Sequences were obtained from all except one of the delimited species in the area under study. In total, ITS was sequenced for 32 species (35 taxa) of *Bupleurum* (31 species sequenced for the first time), including taxa from all the currently accepted sections (*Bupleurum*, *Diaphyllum*, *Reticulata*, *Isophyllum* and *Coriacea*) and subsections of the genus. As an outgroup, two species of another basal genus of *Apioideae*, *Anginon* Raf., were also sequenced for ITS.

A phylogeny derived from cladistic analysis of the aligned ITS sequences clearly shows the division of the genus into two main groups (100% support in both bootstrap and jackknife analyses). This division is also supported by analysis of the 5.8S coding sequence alone. Two new subgenera are proposed: *Bupleurum* and *Penninervia* S.S.Neves *subgenus nov.* Subgenus *Penninervia*, a small basal group, includes all pinnate-reticulate veined species of the genus (*B. angulosum* L., *B. foliosum* Salzm. ex DC., *B. fruticosum* L., *B. gibraltarium* Lam. and *B. stellatum* L.), and also *B. rigidum* L. that has itself a unique type of venation. The association of these species has never been proposed before. Subgenus *Bupleurum*, as defined here, includes all the taxa with typical parallel-veined leaves, and represents the vast majority of the species of the genus. The groups presented within each subgenus are informally treated, because neither morphological or sequence data are conclusive and therefore no formal rank is attributed. The cladistic analysis, including sequences from 4 other basal *Apioideae* genera (*Anginon*, *Heteromorpha* Cham. & Schltdl., *Physospermum* Cusson ex Juss. and *Pleurospermum* Hoffm.), confirms *Bupleurum* as monophyletic.

Molecular data suggests that the endemic Macaronesian species *B. salicifolium* R.Br. ex Buch is a neoendemic, as the sequence divergence between the populations in Madeira and Canary Islands, and closer African taxa is very small. All endemic NW African taxa are included in a single unresolved clade, and the low nucleotide variation of ITS suggests a recent radiation of these taxa. The S African species *B. mundii* is also a neoendemic, which appears closely related to *B. falcatum* L., an Eurasian species.

1. Introduction

1.1 Overview

Bupleurum L., with around 150 species, is one of the largest genera of the *Umbelliferae* family. It includes annual and perennial species, from very small herbs of only a few centimetres (e.g. some specimens of *B. semicompositum* L., or, more surprisingly, *B. ranunculoides* L.) to shrubs of up to 3 metres high (*B. fruticosum* L.). The genus has a high morphological diversity, but is easily distinguished within the family because of an almost unique characteristic: its simple and entire leaves – the only other genus with simple and entire leaves, *Hohenackeria* Fisch. & C.A.Mey., being completely different from *Bupleurum* in its morphology.

Bupleurum has a large distribution in the N Hemisphere, from the W Mediterranean and Macaronesia, throughout Asia to N America (Alaska and Rocky Mountains). There is only one exception to this northern distribution: the S African endemic species *B. mundii* Cham. & Schltdl. Despite this broad distribution, most of the species are rare and restricted to small areas. The highest diversity, morphological or species/taxa number, is found in the Mediterranean, with a large number of endemic species; a high number of taxa is also found in the Himalayan region. *Bupleurum* species grow in a great variety of habitats: from sea level (e.g. *B. tenuissimum* L.) up to 4900 m (the Himalayan *B. longicaule* Wall. ex DC.); through saline marshlands, calcareous, granitic or basaltic soils; in arid or mesophytic places; from open areas to dense forests; as weeds or ruderals; and from equatorial to nearly arctic latitudes (Cauwet, 1976, vol. 1, p. 3-4; Hara & Williams, 1979; Heywood, 1971; Hultén, 1968; Nasir, 1955; Snogerup, 1972).

The following section (1.2) lists the main aims of this project. Explanation of abbreviations used throughout this work is given in section 1.3.

Chapter two presents a critical review of the different taxonomic treatments, which included *Bupleurum* taxa, from the 17th century to the present day. Special relevance is given to the work of Tournefort (1694), the first author that, remarkably for the time, had a concept of the genus that is fundamentally the same as the present delimitation. Presumed relationships of the genus within the family are here also discussed.

A global revision of the genus *Bupleurum* is greatly needed, but such a task is far too broad for a project of this dimension. One of the regions where *Bupleurum* required ample revision was the W Mediterranean, especially NW Africa, which included many inadequately known and rare species. Macaronesian taxa were also revised because of their affinities to Mediterranean taxa. *Chapter 4* presents a brief assessment of the taxonomic problems that were initially found in the various species of the area under study.

When reviewing different classifications is important to discuss the taxonomic value of characters that have been used over the time, and also the potential use of new characters coming from more modern areas of research, all of which could help to clarify the classification and delimitation of taxa (see *chapter 3*). More detailed discussion on character variation in *Bupleurum* is presented in the chapters that are dedicated to particular domains of research (morphology, anatomy, molecular biology).

Most of the morphological study in this work was based on a large collection of herbarium material (> 4,000 specimens) – see *chapter 6*. But the study of living plants provides information that otherwise could not be obtained from dried specimens. Unfortunately, it is not always possible to study the plants in their natural habitats – I was fortunate to have the chance of carrying out field work in the Iberian Peninsula, yet still missed a critical area: NW Africa. Cultivation provides further opportunity of studying living plants and obtaining additional morphological data (e.g. from seedlings). Moreover, it allows the expanding of seed and plant collections in botanical gardens, which are of most importance for conservation. See *chapter 5* for discussion of the cultivation work carried out in this study.

The taxonomic value of the various morphological characters used in *Bupleurum* are generally discussed in *chapter 6*. Further discussion on diagnostic value and variation of particular morphological characters is presented in the species account in *chapter 10* (see critical notes for each species).

Phenetic analysis of 42 characters (40 of which morphological) of the 29 *Bupleurum* species (31 taxa) in the area of study, was carried out (*chapter 6*), and its results are critically discussed and contrasted to those based on molecular analysis.

Fruit characters have been fundamental in the taxonomic investigation of the *Umbelliferae* – see *chapter 7*. The results of anatomical and SEM study of fruits of some species of *Bupleurum* are presented, respectively, in *chapters 7 & 8*.

Molecular studies, in particular DNA sequencing, have had in recent years an enormous impact in taxonomic research. DNA is the genetic material and ultimately the best source of data in the investigation of phylogenetic relationships. Several methods of analysis have been used over the years in molecular systematics (for a review, see introduction in *chapter 9*). Gene sequencing is potentially the most informative method, but selection of genes depends on the taxonomic level under review. The ITS region of nuclear ribosomal DNA includes two fast evolving gene spacers (ITS1 & ITS2) that have been particularly useful to elucidate relationships at the generic and interspecific levels.

ITS was sequenced for 32 species (35 taxa) of *Bupleurum* (31 species sequenced for the first time), including taxa from all the currently accepted sections in the genus; two species of *Anginon* Raf. (endemic to S Africa) were also sequenced – for a total 68 sequenced samples. The results of the phylogenetic analysis of ITS in *Bupleurum* (outgroup = *Anginon*, *Heteromorpha* Cham. & Schltdl., *Pleurospermum* Hoffm. and *Physospermum* Cusson ex Jussieu) and its implications on the classification and delimitation of taxa are amply discussed in *chapter 9* (section 9.4).

A complete taxonomic account of the species of *Bupleurum* in the W Mediterranean and Macaronesia is presented in *chapter 10*. A key to identification of the species that occur in all this area is for the first time produced (section 10.5). This account includes for each of the 29 delimited species in the region, not only detailed morphological descriptions, but also the typification (most of the species required selection of type), synonymy, references to illustrations, chromosome numbers, ecological information, geographical distribution with elucidative maps, and citation of a selection of the herbarium specimens studied. Critical notes on morphological variation (e.g. pointing out the characters that are more useful for identification), nomenclature, typification, conservation and plant uses, are also included.

Finally, *chapter 11* summarises the main conclusions on species delimitation and phylogenetic relationships in *Bupleurum*, particularly in the W Mediterranean and Macaronesia, which resulted from the investigation of the different sources of characters. Emphasis is given to the taxa that most need further research.

1.2 Aims of this study

- To present a critical review of the taxonomic history of *Bupleurum*.

- To study the morphology of the species/taxa of *Bupleurum* in the W Mediterranean and Macaronesia, searching for useful diagnostic characters and those that may be relevant in the classification.

- To revise nomenclature and synonymy of the taxa in this area.

- To check typification or typify the *Bupleurum* species/taxa in the W Mediterranean and Macaronesia.

- To enlarge collections of herbarium and fruit material of *Bupleurum*.

- To cultivated the largest possible number of taxa of *Bupleurum* to study morphology in general, and in particular seedling features.

- To expand living collections of *Bupleurum* in Botanic Gardens, in particular at the Royal Botanic Garden Edinburgh and the Jardim Botânico de Coimbra (Portugal).

- To look for potentially useful characters from anatomy and SEM study of fruits.

- To produce the account of the genus *Bupleurum* and its species for the *Flora iberica* project (taxonomic revision of Iberian and Balearic species) including keys to identification, full descriptions and other information such as ecology, detailed distribution and critical observations.

- To sequence an informative gene to find evidence of possible phylogeny of the genus *Bupleurum*.

- To discuss the possible evolutionary relationships in *Bupleurum*, especially in the W Mediterranean and Macaronesia.

- To look for the closest genera to *Bupleurum*.

- To revise the taxonomy of all the species of the W Mediterranean and Macaronesia and present information on types, synonymy, morphology, chromosome numbers, ecology, detailed distribution and critical taxonomic notes.

1.3 Abbreviations used

Most of the abbreviations used are standard (see e.g. Stearn, 1983, p. 367-372), and very familiar to botanists, and biologists in general. Nevertheless, for clarity explanation is given below.

General abbreviations:

acc. – accession
al. – *alii*: others
alt. – altitude
auct. – *auctorum*: of authors
auct. non – authors other than
C – Central
c., ca. – *circa*: about, near
Cat. – catalogue
cf. – *confer*: compare
cl., coll. – collector
Co. – company
cont. – continuation or continuing
E – East
e.g. – *exempli gratia*: for example
ed. – edition or editor
eds – editors
Exped. – expedition
f. – (after a personal noun) *filius*: son
fig. – figure, illustration
figs – figures
hab. – habitat, place of growth
herb. – herbarium
i.e. – *id est*: that is
IDC – Inter Documentation Company bv (Leiden, The Netherlands)
ined. – *ineditus*: unpublished
isl. – islands
lib. – *liber*: book
m.s.m. – *metra supra mare*: metres above sea level
N – North
n°, no, no. – *numero*: number
nom. – *nomen*: name
nom. inval. - *nomen invalidum*: invalid name
Nos – numbers
nov. – *novus*: new
p. – page, pages
p.p. – *pro parte*: partly, in part
pro syn. – *pro synonymon*: as a synonym
prov. – province
q.v. – *quod vide*: which see (e.g. showing a cross-reference)
rpm – revolutions per minute
S – South
SEM – Scanning Electron Microscope
s.l. – *sensu lato*: in a broad sense
s.n. – *sine numero*: without a number
s. str. – *sensu stricto*: in a narrow sense
sect. – section
sp. – species
spp. – species (plural)
ssp. – subspecies
subsect. – subsection
subsp. – subspecies
syn. – synonym
tab. – *tabula*: plate
Univ. – University
UV – ultraviolet [light]
v., var. – variety
vol. – volume
vols – volumes
W – West

Symbols & units:

± more or less

= the same as, synonym of

! – (after herbarium abbreviation or icon) seen by myself

i - xii – (in herbarium citations) months of the year

A – ampere(s) [electric current]

°C – degree(s) celsius (centigrade)

M – molar: moles per litre [e.g. 1M = 1 mole of solute per 1 litre (1000g) of solvent]

mol – mole; moles of different elements or compounds contain the same number of molecules = 6.023×10^{23} (Avogadro number)

S – Svedberg (sedimentation coefficient) [e.g. the 'S' in the 5.8S or 26S – subunits of rDNA]

V – volt(s) [electric potential]

hr – hour(s)
min – minute(s)
sec – second(s)

g – gram(s)
l – litre(s)

m – metre(s)
cm – centimetre(s) (1×10^{-2} m)
mm – millimetre(s) (1×10^{-3} m)

m- – milli- (10^{-3}) [e.g. ml or mg]
μ- – micro- (10^{-6}) [e.g. μm or μl]
n- – nano- (10^{-9}) [e.g. nm or ng]
p- – pico- (10^{-12}) [e.g. pmol]

n – haploid chromosome number
2n – diploid chromosome number
x – basic chromosome number

Molecular abbreviations:

AFLPs – amplified fragment length polymorphisms

bp – base pair(s)

CTAB – hexadecyltrimethyl- (or cetyltrimetyl-) ammonium bromide

ddNTPs – dideoxynucleosides triphosphates [ddATP, ddCTP, ddGTP, ddTTP]

DNA – deoxyribonucleic acid

- **cpDNA** – chloroplast DNA
- **mtDNA** – mitochondrial DNA
- **rDNA** – ribosomal DNA
- **nrDNA** – nuclear ribosomal DNA

dNTPs – deoxynucleoside triphosphates [dATP, dCTP, dGTP, dTTP]

DTT – dithiothreitol

EDTA – ethylenediaminetetra-acetic acid

IAA – isoamyl alcohol

ITS – *internal transcribed spacers*: two gene spacers (ITS 1 and ITS 2) located between the 18S and 26S (25S or 28S) subunits of nuclear ribosomal DNA (eukaryotes). *ITS region* includes ITS 1 and ITS 2, and the 5.8S subunit located between the two spacers.

kb – kilobase pairs (1000 bp)

PCR – polymerase chain reaction

RAPDs – randomly amplified polymorphic DNAs
REs – restriction endonucleases
RFLPs – restriction fragment length polymorphisms
RNA – ribonucleic acid
mRNA – messenger RNA
rRNA – ribosomal RNA
SSRs – simple sequence repeats
STRs – short tandem repeats
TBE – Tris-boric acid-EDTA buffer used for electrophoresis of DNA
TE – Tris-EDTA buffer used to dilute DNA
Tris – Trizma; 2-amino-2-(hydroxymethyl)-1,3-propanediol
VNTRs – variable number of tandem repeat loci

Nucleotides:

IUB Codes: (IUB = International Union of Biochemistry)

A – adenosine
C – cytidine
G – guanosine
T – thymidine

R – **A** or **G** (puRine)
Y – **C** or **T** (pYrimidine)
K – **G** or **T** (Keto)
M – **A** or **C** (aMino)

S – **G** or **C** (Strong -3H bonds) [H = hydrogen bond]
W – **A** or **T** (Weak -2H bonds)
N – aNy base

Other abbreviations:

Abbreviation of titles of books in chapter 10 follows, when possible, Stafleu (1967), Stafleu & Cowan (1976-1988), and Stafleu & Mennega (1992-1998). Also in chapter 10, abbreviation of journals follows Lawrence *et al.* (1968), and Bridson (1991). Authors names are abbreviated according to Brummit & Powell (1992). See also section 10.2.3, for abbreviations of names of geographical regions, provinces or islands used in the text. For standard abbreviations of herbaria see Appendix I.

2. Taxonomic History

2.1 Ancient history

The word 'Bupleurum', from the Greek βουπλευρον [*bous* (ox) and *pleuron* (side, rib)], was used in the works of Greek and Roman philosophers such as Theophrastus of Eresos (c. 370-285 BC) in his *Historia Plantarum* (see 1644 ed.) and Pliny the Elder (23-79 AD) in his *Di Historia Mundi* (see 1610 ed.). From the meaning of this word, 'ox rib' or 'ox side', we may assume that the name was applied to plants with hard textured (coriaceous) leaves or stems. Although Theophrastus' description of 'Bupleurum' might correspond to an umbellifer, it is doubtful that the term was then used to refer to plants now accepted as *Bupleurum*.

According to Sprengel (1813a) another Greek name, 'Buprestis' (βουπρεστις = 'ox burner'), was used by Theophrastus and Hippocrates (c. 460-377 BC) to name plants akin to *Bupleurum* (see also Burtt, 1991). But there was some confusion in the use of this word. 'Bouprestis' was also used by Dioscorides (40-80 AD), in *De Materia Medica* (see: Gunther, 1934; Matthioli, 1562), yet he did not refer to *Bupleurum* plants or to any plant at all, but rather to a species of beetle, the blister beetle (Riddle, 1985).

The Greek vernacular names survived for centuries as they were kept in use by the followers of these early botanists. But, as often happens with the common names of plants, some of them lost their original meaning, no longer referring to the same plants, the likely case in *Bupleurum*.

2.2 The circumscription of the genus

In the 17th century, various botanists used the name *Bupleurum* to cite and describe taxa of the genus in its present sense, and often these plants were correctly placed among other umbellifers. Nevertheless, most of these authors placed the taxa we recognize now as *Bupleurum* in different genera.

Caspar (Kaspar or Gaspar) Bauhin in his *Pinax Theatri Botanici* (1623) placed the taxa of *Bupleurum* in three different 'genera': *Perfoliata*, *Bupleurum* and

Seseli. *Perfoliata* and *Bupleurum* appear alongside each other, but separated from the rest of the umbellifers. The taxon cited as '*Seseli Aethiopicum salicis folio*' (a pre-Linnaean polynomial or phrase name), corresponds to *Bupleurum fruticosum* L., and this one was indeed placed in the umbellifers.

Jean Bauhin (Caspar's older brother) and J. Cherler, in their *Historia Plantarum Universalis* (1651), placed the *Bupleurum* taxa in 3 *Umbelliferae* 'genera': *Seseli*, *Perfoliata* and *Auricula*. But all these taxa were presented in succession, as if they were believed related. Even '*Seseli aethiopicum Fruticosum*' (*B. fruticosum* L.) was separated from the rest of the taxa of *Seseli* and placed beside *Perfoliata* and *Auricula*. 'Bupleuron' is here only referred to as an old name used by the ancient philosophers ('Bupleuron Veteribus').

Robert Morison in his *Plantarum Umbelliferarum Distributio nova* (1672) described the *Bupleurum* taxa in two 'genera': *Perfoliata* and *Bupleurum*. However, he recognized them as closely related taxa forming the group described as "umbellae foliis quid peculiare" (umbels with peculiar leaves). Morison obviously realized how unusual in the family are the simple, entire leaves of *Bupleurum*, and he cited the character in his description.

John Ray included *Bupleurum* in his major work, *Historia Plantarum* (1686), but placed some of the taxa in two other 'genera', *Perfoliata* and *Auricula*, giving, once again, greater importance to the type of insertion of the [upper] leaves (perfoliate, auriculate). But, he too, classified them as a group named "De Plantis Umbelliferis foliis simplicibus" (umbels with simple leaves).

The concept of *Bupleurum* as a genus is due to Joseph Pitton Tournefort in his *Élémens de Botanique* (1694), where he assembled a representative number of species nowadays included in the genus. He described *Bupleurum* using the morphology of the flower and fruit, and emphasized the presence of entire leaves. Quite remarkably, Tournefort included in *Bupleurum* species from all currently accepted sections (*Bupleurum*, *Diaphyllum*, *Reticulata*, *Isophyllum* & *Coriacea*) and subsections, and none from different genera – see pages 11 & 12 for a list of these taxa. Fig. 2.1 (p. 10) shows the extract where Tournefort described his 'species' of *Bupleurum*.

GENRE XI.

Buplevrum.

LA Percefeuille eſt un genre de plante dont la fleur A eſt ordinairement à cinq feuilles B diſpoſées en roſe à l'extremité du calice C. Lorſque la fleur eſt paſſée ce calice devient un fruit D compoſé de deux graines E F oblongues, arondies ſur le dos G & canelées. Ajoutez au caractere de ce genre les feuilles ſimples & alternes. Pl. 161.

Les eſpeces de Percefeuille ſont,

[1] Buplevrum folio rigido C.B. pin. 278.
[2] Bupleyrum anguſtifolium herbariorum Lob. Ic. 456.
[3] Buplevrum anguſtiſſimo folio C.B. pin. 278.
[4] Bupleyrum annuum anguſtifolium Bot. Monſp.
[5] Buplevrum perfoliatum rotundifolium annuum. *Perfoliata vulgatiſſima ſive arvenſis C.B. pin. 277.*
[6] Buplevrum perfoliatum longifolium annuum. *Perfoliata annua longioribus foliis J. B. 3.198.*
[7] Buplevrum montanum latifolium. *Perfoliata montana latifolia C.B. pin. 277.*
[8] Buplevrum montanum floſculis exiguis. *Perfoliata montana floſculis parvis C.B. pin. 277.*
[9] Buplevrum Alpinum latifolium minus. *Perfoliata Alpina latifolia minor. C.B. pin. 277.*

R ij

[10] Buplevrum Alpinum anguſtifolium majus. *Perfoliata Alpina anguſtifolia major, ſive folio anguloſo C.B. pin. 277.*
[11] Buplevrum Alpinum anguſtifolium medium. *Perfoliata Alpina anguſtifolia media C.B. pin. 277.*
[12] Buplevrum Alpinum anguſtifolium minus. *Perfoliata Alpina anguſtifolia minor C.B. pin. 277.*
[13] Buplevrum montanum anguſtifolium. *Perfoliatum anguſtifolium montanum Col. part. 1. 247.*
[14] Buplevrum arboreſcens Salicis folio. *Seſeli Æthiopicum Salicis folio C.B. pin. 161.*
[15] Buplevrum Hiſpanicum arboreſcens gramineo folio.
[16] Buplevrum Hiſpanicum fruticoſum aculeatum gramineo folio.
[17] Buplevrum Luſitanicum gramineo, longiori, & rigidiſſimo folio.

Fig. 2.1 – Tournefort description of the 'species' of *Bupleurum*. Extract of *Élémens de Botanique* (1694). The numbers in square brackets are not in the original text, they indicate the different taxa (see p. 11 & 12). In the text, '*C.B. pin.*' refers to C. Bauhin *Pinax* (1623); '*Col.*' is Colonna or Columna (1606); '*J.B.*' is J. Bauhin & Cherler (1651); '*Lob. Ic.*' is Lobel (or L'Obel) *Plantarum seu Stirpium Icones* (1581); and '*Bot. Monſp.*' is Magnol's *Botanicum Monspeliense* (1676).

Below are listed the modern names of *Bupleurum* taxa corresponding to the phrase names of Tournefort (see Fig. 2.1, p. 10). I have seen his herbarium in Paris (P-TRF) – Muséum National d'Histoire Naturelle (Tournefort herbarium is also available in microfiche) – and revised the references cited in his text (C. Bauhin, 1623; J. Bauhin & Cherler, 1651; Colonna or Columna, 1606; Lobel or L'Obel, 1581; & Magnol, 1676). The handwriting on the specimens of Tournefort seems to be his own – see photo of a specimen in Fig. 2.2, p. 13 (cf. facsimile of Tournefort' handwriting in Dandy, 1958).

The *Bupleurum* taxa of Tournefort, *Élémens de Botanique* (1694)

[1] '*Bupleurum folio rigido*' = ***Bupleurum rigidum*** L. subsp. ***rigidum*** [Sect. *Isophyllum* (Hoffm.) Dumort, Subsect. *Marginata* (Gren. & Dodr.) H.Wolff] – See illustrations in Lobel (1581, p. 244) and Daléchamps (1586-1587, p. 741), both cited by C. Bauhin (1623).

[2] '*Bupleurum angustifolium herbariorum*' = ***B. praealtum*** L. [Sect. *Isophyllum* (Hoffm.) Dumort., Subsect. *Juncea* Briq.] – See Lobel (1581, p. 456) and compare to Dodoens (1616, p. 633). The illustration of Dodoens is the neotype of *B. praealtum* L. (Snogerup, 1972). C. Bauhin (1623) confused this species with *B. falcatum* L. Tournefort was aware of the difference as he cited only Lobel, and not C. Bauhin as he did for the other taxa. This is confirmed in Tournefort's *Institutiones Rei Herbariae* (1700) were he uses the recognizable phrase name '*Bupleurum folio subrotundo, sive vulgatissimum*' to refer to *B. falcatum* L – see also Tournefort specimen 2710 (P-TRF).

[3] '*Bupleurum angustissimo folio*' = ***B. tenuissimum*** L. [Sect. *Isophyllum* (Hoffm.) Dumort, Subsect. *Trachycarpa* (Lange) Briq.] – See Colonna (1606, p. 247) cited by C. Bauhin (1623), and also Tournefort specimen 2713 (P-TRF).

[4] '*Bupleurum annuum angustifolium*' = ***B. gerardii*** All. [Sect. *Isophyllum* (Hoffm.) Dumort., Subsect. *Juncea* Briq.] – Magnol's '*Bupleurum annuum angustifolium*' corresponds to *B. praealtum* L. (see also Stearn, 1973). However, Tournefort apparently thought it referred to the taxon we recognize now as *B. gerardii* All. – See specimen 2711 (P-TRF).

[5] '*Bupleurum perfoliatum rotundifolium annuum*' = ***B. rotundifolium*** L. [Sect. *Bupleurum*] – See Dodoens (1583, figure in p. 104) cited by C. Bauhin (1623).

[6] '*Bupleurum perfoliatum longifolium annuum*' = ***B. lancifolium*** Hornem. [Sect. *Bupleurum*] – See illustration and description in J. Bauhin & Cherler (1651, p. 198).

[7] '*Bupleurum montanum latifolium*' = ***B. longifolium*** L. [Sect. *Diaphyllum* (Hoffm.) Dumort.] – See Tournefort specimen 2714 (P-TRF) – photo in Fig. 2.2, p. 13. The phrase name '*Perfoliata montana* Cam.' is cited by C. Bauhin (1623).

[8] '*Bupleurum montanum flosculis exiguis*' = ? – No description or illustration is indicated.

[9] '*Bupleurum Alpinum latifolium minus*' = ? – Insufficient information in the description of C. Bauhin (1671).

[10] '*Bupleurum Alpinum angustifolium majus*' = ***B. angulosum*** L. [Sect. *Reticulata* Gren. & Godr.] – See description in C. Bauhin (1671, p. 129).

[11] '*Bupleurum Alpinum angustifolium medium*' = ***B. stellatum*** L. [Sect. *Reticulata* Gren. & Godr.] – See description in C. Bauhin (1671, p. 129).

[12] '*Bupleurum Alpinum angustifolium minus*' = ***B. ranunculoides*** L. [Sect. *Isophyllum* (Hoffm.) Dumort., Subsect. *Nervosa* (Gren. & Godr.) Briq.] – See description in C. Bauhin (1671, p. 129-130).

[13] '*Bupleurum montanum angustifolium*' = ***B. odontites*** L. [Sect. *Isophyllum* (Hoffm.) Dumort., Subsect. *Aristata* (Gren. & Godr.) Briq.] – See illustration in Colonna (1606, p. 247).

[14] '*Bupleurum arborescens Salicis folio*' = ***B. fruticosum*** L. [Sect. *Coriacea* Gren. & Godr.] – See Dodoens (1583, p. 310) and Daléchamps (1586-1587, p. 750), both cited by C. Bauhin (1623).

[15] '*Bupleurum Hispanicum arborescens gramineo folio*' = ***B. fruticescens*** L. subsp. ***fruticescens*** [Sect. *Isophyllum* (Hoffm.) Dumort., Subsect. *Rigida* (Drude) H.Wolff] – See Tournefort specimen 2717 (P-TRF).

[16] '*Bupleurum Hispanicum fruticosum aculeatum gramineo folio*' = ***B. fruticescens*** L. subsp. ***spinosum*** (Gouan) O.Bolós & Vigo [Sect. *Isophyllum* (Hoffm.) Dumort., Subsect. *Rigida* (Drude) H.Wolff] – See specimen 2718 (P-TRF).

[17] '*Bupleurum Lusitanicum gramineo, longiori, & rigidissimo folio*' = ***B. rigidum*** L. subsp. ***paniculatum*** (Brot.) H.Wolff [Sect. *Isophyllum* (Hoffm.) Dumort., Subsect. *Marginata* (Gren. & Godr.) H.Wolff] – See specimen 2719 (P-TRF).

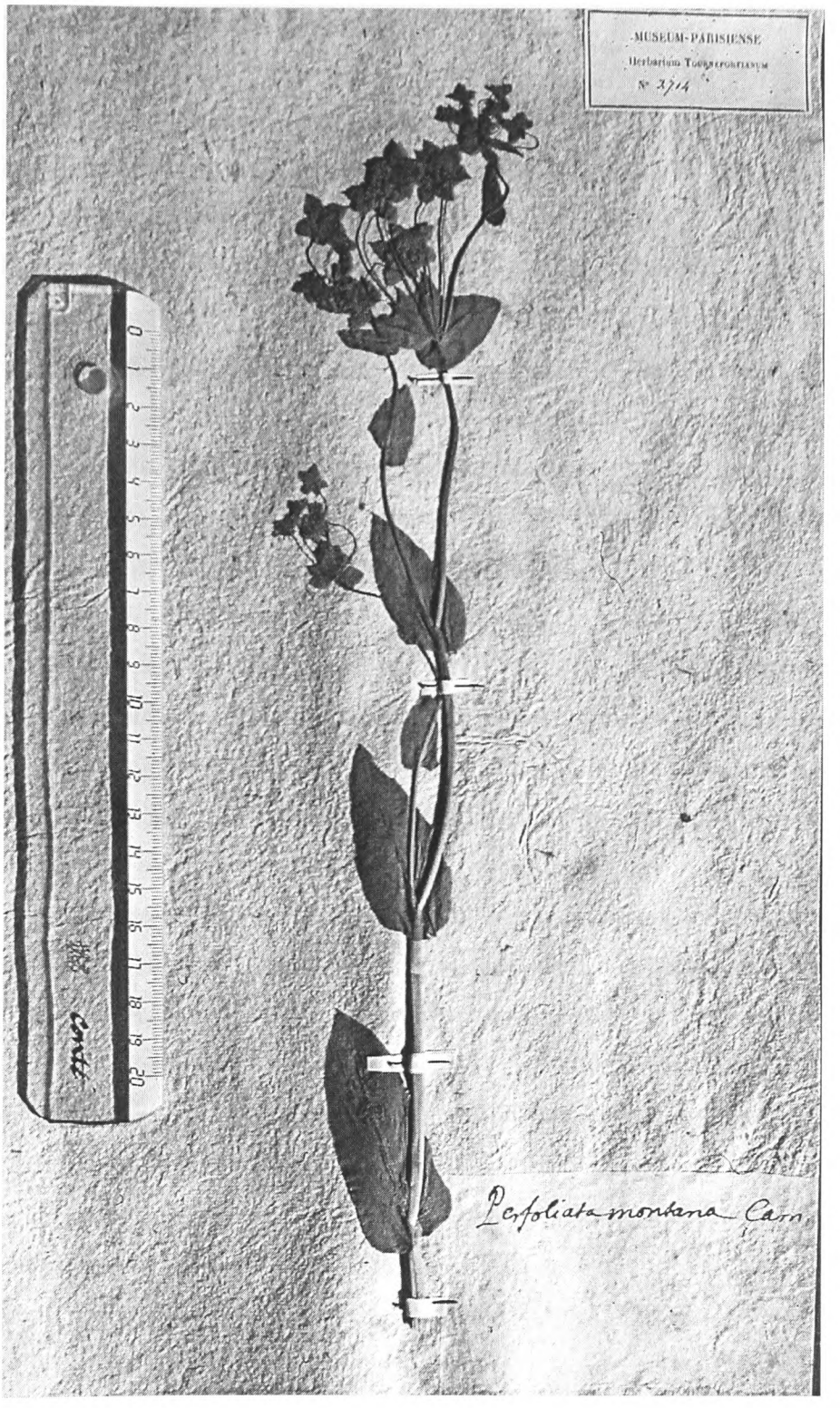

Fig. 2.2 – '*Perfoliata montana* Cam.' (= *Bupleurum longifolium* L.) in the Tournefort herbarium. Specimen 2714 (P-TRF) – Paris, Muséum National d'Histoire Naturelle. Scale is in cms. This specimen is c. 300 years old, but, like the others in the Tournefort collection, is in a remarkable good condition.

2.3 From Linnaeus to the present

Considering the morphological diversity found in *Bupleurum,* it is not surprising that many of these plants were initially classified in different genera: *Buprestis* Spreng, 1813(a); *Diaphyllum* Hoffm., 1816; *Diatropa* Dumort., 1827; *Isophyllum* Hoffm., 1816; *Odontites* Spreng., 1813(a); *Perfoliata* Fourr., 1868; *Tenoria* Spreng., 1813(b); *Trachypleurum* Rchb., 1828; and others (see Pimenov & Leonov, 1993; for the particular cases of *Buprestis* and *Tenoria*, see also Burtt, 1991).

Linnaeus did recognize all these plants as a single genus, *Bupleurum*, which he integrated in his sexual system in *Systema Naturae* (1735) and *Species Plantarum* (1753): classified in Class V: *Pentandria*, Order *Digynia*.

In *Species Plantarum* Linnaeus included 13 species of *Bupleurum*: *B. rotundifolium*, *B. stellatum*, *B. petraeum*, *B. angulosum*, *B. longifolium*, *B. falcatum*, *B. odontites*, *B. ranunculoides*, *B. rigidum*, *B. tenuissimum*, *B. villosum*, *B. fruticosum*, *B. difforme*. All of these are still accepted as valid species, but two of them are now placed in different genera: *Bupleurum villosum* L. = *Hermas villosa* (L.) Thunb., and *Bupleurum difforme* L. = *Anginon difforme* (L.) B.L.Burtt (see Burtt, 1991). Linnaeus did not fully recognize the significance of the simple and entire leaves and the absence of hairs in *Bupleurum*; both *Hermas* and *Anginon* have divided leaves, and *Hermas villosa*, as the specific name implies, has hairs.

The taxonomic history of *Bupleurum* is summarized in Table 2.1, p. 22. Linnaeus only subdivided the genus into herbs (*Herbacea*) and shrubs (*Frutiscentia*) and for that reason does not appear in Table 2.1.

Later authors such as Sprengel (1813a,b; 1820), Hoffmann (1816), Berchtold & Presl (1820), Dumortier (1827, 1829), L. Reichenbach (1828) and Calestani (1905) included *Bupleurum* species in various other genera (Table 2.1).

W.D.J. Koch (1824) recognised all these genera (*Bupleurum* L., *Diaphyllum* Hoffm., *Isophyllum* Hoffm., *Odontites* Hoffm. and *Tenoria* Spreng.) as a single genus *Bupleurum* which he describes as '*Bupleuri genus naturalissimum*' – Koch was very confident about considering the genus as a natural group. This author also used the morphological characters of the petals to distinguish different genera. Koch's work is not included in Table 2.1, as he did not subdivide the genus.

Grenier & Godron (1848) subdivided *Bupleurum* for the first time in six sections (*Perfoliata, Reticulata, Nervosa, Aristata, Marginata* and *Coriacea*) using the following characters: absence/presence and direction of bracts; insertion of leaves; venation type and vein number on leaves. Each section was characterized by at least two characters. Boissier (1872) had a similar concept of the genus, although he preferred to consider sections *Nervosa* and *Marginata* as just one section, *Graminea*. Boissier used a few additional characters to describe his sections: habit, and width and texture of bracteoles.

Briquet (1897), in a monograph on the alpine species, followed the groups of Grenier & Godron, but placed *Aristata*, *Nervosa* & *Marginata* in just one section, *Eubupleura*. Briquet used a larger number of characters, including data from anatomy of stems, leaves and fruits; he presented both keys for both morphological and anatomical characters.

Drude (1898) basically followed the classification of Briquet, but proposed a new section, *Rigida*, including species from the groups *Nervosa* and *Marginata*.

Calestani (1905), although accepting the groups of Grenier & Godron and Briquet, again split *Bupleurum* into two genera, resuscitating *Trachypleurum*. This genus was based on just one character (rough fruit) and even its own author, L. Reichenbach (1828), included it later in *Bupleurum* (Reichenbach, 1830-32).

Wolff's taxonomic revision of *Bupleurum* (Wolff, 1910), integrated the classifications of Grenier & Godron (1848), Briquet (1897) and Drude (1898); he also proposed a new section, *Longifolia*, with some of the species of Sect. *Reticulata*. Wolff produced extended descriptions of groups and species including all the characters used by previous authors. Wolff also used morphological characters of petals to distinguish different *Bupleurum* taxa. However, despite the remarkable volume work presented by Wolff, a major criticism has to be pointed out: an excessive subdivision of *Bupleurum* into infraspecific taxa. He created an indefinite number of artificial varieties, subvarieties and forms for many of the species.

Koso-Poljansky (1913), in the first part of his *Epitome Bupleurorum Rossiae* (the Russian species of *Bupleurum*), presented a taxonomic history of the genus and proposed a significantly modified new classification of *Bupleurum*. He divided the genus for the first time in three subgenera using mainly habit and involucre and involucel characters: *Diatropa* with 2 sections; *Bupleurotypus* with 3 sections; and

Agostana with 2 sections (see Table 2.1, p. 22). Subgenus *Diatropa* (Dumort.) Koso-Pol. (= Sect. *Perfoliata* Gren. & Godr. – annual herbs with perfoliate upper leaves and no bracts) is a fairly natural group, but does not seem to merit such a high rank in the classification. Subgenus *Bupleurotypus* Koso-Pol. formed by all the perennials of the genus is a very artificial group, because only one character was really taken into account: the perennial habit. Subgenus *Agostana* (S.F.Gray) Koso-Pol., including only annuals with bracts and no perfoliate leaves, is probably a paraphyletic group, because some of the perennials with similar characters should have been included.

Cerceau-Larrival (1962, p. 142) proposed a considerably modified classification of *Bupleurum* (not included in Table 2.1 for practical reasons), which was based on (peculiarly defined) leaf characters. She divided the genus into two sections: 1) "Section I = *Graminifolia*" ("leaves without secondary veins"), essentially corrresponds to Sect. *Isophyllum*, and so this new name is superfluous; and 2) "Section II" [*Bupleurum*] ("leaves with secondary veins"), which included the remaining taxa. The latter section was subdivided into two subsections: 1) Subsect. *Reticulata* [including all the species with pinnate-reticulate leaves, but also taxa with parallel-veined leaves: *B. longifolium* and '*B. paniculatum*' (= *B. rigidum* subsp. *paniculatum*)]; and 2) Subsect. *Perfoliata*. However, Cerceau-Larrival included in Sect. *Graminifolia* species that sometimes (or often!) present slender secondary veins between the main parallel veins, e.g. *B. ranunculoides* (specimens with broader leaves) and *B. falcatum*. Therefore, this sectional division of the genus is highly artificial. The author also associated *B. fruticosum* to '*B. paniculatum*' apparently because they both have 'une nervation marginale'; a thick intramarginal vein is indeed present in the latter taxon, but there is no such vein in *B. fruticosum* (all leaf characters of these two taxa are very distinct).

The classification adopted by Tutin in *Flora Europaea* (1968) is currently the most generally accepted classification of the genus. It basically follows that of Wolff (1910). The main modifications are the nomenclature of the sections, and the elimination of the subsectional rank in Sect. *Bupleurum* (= Sect. *Perfoliata* Gren. & Godr.) – see Table 2.1. The change in the nomenclature was needed because, as the International Code of Botanical Nomenclature (ICBN, 1994) states, the name assigned to the section containing the type species (in this case *B. rotundifolium* L. – see *Taxon* 41: 572, 1992; 44: 612, 1995) has to be the same as that of the genus (Art.

22.1). Also Sect. *Diaphyllum* (Hoffm.) Dumort. is an earlier name for Sect. *Longifolia* H.Wolff. Furthermore, names formed by adding the prefix *Eu-* (like in Sect. *Eubupleura* Briq.) are not accepted under the present Code (Art. 21.3).

The most recent taxonomic revision of the genus was carried out by Cauwet in her PhD thesis (1976), where she proposed the division of the genus into two subgenera: *Bupleurum* with 4 sections, and *Tenoria* (Spreng.) Cauwet with 2 sections (see Table 2.1, p. 22). Nevertheless, this new classification is questionable as not even a single character was indicated to support the separation of these new subgenera. Furthermore, Cauwet's cladistic analysis, on which she based her final taxonomic decisions concerning the subgenus *Tenoria* (the main subject of her thesis), did not include any of the annual *Bupleurum* species, from the proposed subgenus *Bupleurum*, in spite of including all the perennials of the two new subgenera, as well as species from other genera. The reason presented for this exclusion was "the systematic definition of the subgenus *Tenoria*" which is that all species in subgenus *Tenoria* are perennial (Cauwet, 1976 - 1: 274; 3: 19, 38) – hardly a satisfactory explanation for introducing a new taxonomy.

In summary, after Grenier & Godron (1848), there has been no general agreement on the groups status as the species have been classified either as sections, subsections or even subgenera in the latest works. Also, several species have been placed in different groups in the subsequent classifications (e.g. *B. falcatum* L. and *B. rigidum* L.). The sections *Bupleurum* (= Sect. *Perfoliata* Gren. & Godr., e.g. *B. rotundifolium* L.) and *Coriacea* Gren. & Godr. (e.g. *B. fruticosum* L.) are the exceptions as they have shown a more stable classification. The classifications of Koso-Poljansky (1916), Cerceau-Larrival (1962) and Cauwet (1976) are the more discordant. Koso-Poljansky and Cauwet gave too much weight to habit as a character; while Cerceau-Larrival used a few erroneously defined leaf characters.

Although there is now a significant amount of information about many of the *Bupleurum* species, the taxonomy of the genus is far from being satisfactory and considerable research is still needed. This is the main reason for the present research. Several species of the so called Sect. *Isophyllum* (Hoffm.) Dumort (many of which are endemic to the area in the present study) require extended revision; some of them are: *B. acutifolium* Boiss., *B. atlanticum* Murbeck, *B. balansae* Boiss., *B. canescens* Schousb., *B. handiense* (Bolle) G.Kunkel, *B. montanum* Coss. and *B. oligactis* Boiss.

2.4 Suprageneric classification of *Bupleurum*

Considering suprageneric classifications, the genus *Bupleurum* has in the past been considered at the rank of family (*Bupleuraceae* Spreng. ex Pfeiffer, 1873), although other authors have treated it at lower rank: subfamily (*Bupleuroideae* Cerceau-Larrival, 1962); tribe (*Bupleureae* Spreng., 1820 - '*Bupleurineae*'); and subtribe (*Bupleurinae* Spreng. ex Koso-Pol., 1916) – see Pimenov & Constance, 1985.

Nowadays the most generally accepted classification is: Family *Umbelliferae* / *Apiaceae*; Subfamily *Apioideae*; Tribe *Apieae*; Subtribe *Apiinae* (including other major genera such as *Pimpinella* L. and *Carum* L.) – Heywood, 1971.

Downie *et al.* (2000) presented a revision of the classification of the *Umbelliferae* family, and according to them, strong evidence (several molecular studies) suggests that *Bupleurum* forms a quite distinct group in the *Umbelliferae* and should have the status of Tribe *Bupleureae*, as first used by Sprengel (1820) but including only one genus.

2.5 The relationships of the genus *Bupleurum*

Several genera have been associated with *Bupleurum*. Drude (1898), later followed by H.Wolff (1910), placed *Bupleurum* in the *Ammineae-Carinae Heteroclitae* which include the following other genera (present nomenclature is used – see Pimenov & Leonov, 1993):

- *Anginon* Raf. (Syn. *Rhyticarpus* Sond.) – subshrubs; endemic to S Africa.
- *Ferula* L. p.p. (Syn. *Buniotrinia* Stapf & Wettst.) – perennial herb; Middle East.
- *Heteromorpha* Cham. & Schltdl. – small trees or shrubs; Africa (NE, tropical, S) and SW Asia (Yemen).
- *Hohenackeria* Fisch. & C.A.Mey. – perennial herbs; SW Europe, NW Africa and SW Asia and the Caucasus.
- *Glia* Sond. (Syn. *Ruthea* Bolle p.p.) – shrub; endemic to S Africa.
- *Ledebouriella* H.Wolff – perennial herbs; Middle East & C Asia

- *Lichtensteinia* Cham. & Schltdl. – perennial herbs; endemic to S Africa and St Helena.
- *Nirarathamnos* Balf.f. – subshrub; endemic to the island of Socotra (NE Africa).
- *Rutheopsis* A.Hansen & G.Kunkel (Syn. *Ruthea* Bolle p.p.) – perennial herbs; NW Africa and Canary Islands.
- *Trinia* Hoffm. – perennial herbs; broad distribution in Europe and Asia.

The reason for the grouping in the *Ammineae Heteroclitae* is not clear, as not a single common character was indicated – the meaning of the word *Heteroclitae* is also cryptic: from the Greek, '*hetero*' (= different), and maybe '*clitos*' (= sinking, drooping, concluding). However, this grouping might possibly refer to Drude's and Wolff's view of these genera as relics ('reliquae') in the *Ammineae*, i.e primitive genera.

Koso-Poljansky (1916) proposed a very artificial classification of the *Umbelliferae* based solely on fruit characters. He divided the family into two subfamilies, and placed *Bupleurum* in the *Hydrocotyloideae*. According to him, *Bupleurum* would form a group with *Anethum* L. (origin SW Asia?; largely cultivated), *Nirarathamnos*, *Pyramidoptera* Boiss. (Afghanistan) and *Rhyticarpus* (= *Anginon*), because these genera share [only] one character: calcium-oxalate crystals are absent in the pericarp or were 'not seen'!

Cerceau-Larrival (1962), using pollen and seedling characters, indicated another genus, *Hermas* L. (perennial herbs; endemic to S Africa), as closely related to *Bupleurum*, and classified both genera as subfamily *Bupleuroideae*.

However, the affinities of these genera are supported by very few characters, frequently just one (e.g. entire leaves, between *Hohenackeria* and *Bupleurum*). Cauwet (1976) did not find any evidence of a close relationship as she concluded that "if there are phyletic lines between these genera, they are excessively loose and difficult to identify" (Cauwet, 1976 - 1: 294).

Bupleurum is one of the few *Umbelliferae* genera with woody species: shrubs (e.g. *B. fruticosum* L. and *B. salicifolium* R.Br.) and subshrubs (e.g. *B. fruticescens* L. and *B. dumosum* Coss. & Balansa). There are about 10 other woody genera in the family (Burtt, 1991) – the *Umbelliferae* have over 400 genera (Mabberley, 1997).

The woody habit is sometimes regarded as a primitive state in the family; this is inferred by the close relationship to the *Araliaceae*, which are mostly woody. However, secondary woodiness is believed to occur in the *Umbelliferae*, a good example of which seems to be *Nirarathamnos*, according to Alexei Oskolski of the Botanical Museum of St. Petersburg (personal communication).

Considering the rarity of the woody habit in the family, the closest allies of *Bupleurum* are likely to be found among these woody genera (almost all African), such as: *Anginon*, *Glia*, *Heteromorpha*, *Marlothiella* H.Wolff, *Nirarathamnos*, *Polemannia* Eckl. & Zeih. and *Polemanniopsis* B.L.Burtt.

But habit alone is not enough, some other characters need to be considered. For instance, *Nirarathamnos* and *Polemanniopsis* are unlikely allies of *Bupleurum* as the only clear common character between the genera is of the woody state.

Heteromorpha [e.g. *H. trifoliata* (Wendl.) Eckl. & Zeyh.] has, among other differences, very distinct flowers and fruits, but the venation and the marginal stripe of the leaves (leaflets) are reminiscent of the features of *Bupleurum fruticosum* and *B. gibraltarium*, as also are the reflexed bracts of the latter. *Polemmannia* seems to be related to *Heteromorpha*, and has the same type of venation. But, this type of reticulate venation could be a plesiomorphy (primitive shared feature) in the family. Then, it may be more widespread than I can ascertain and, therefore, not really useful when establishing relationships. Plesiomorphies normally do not constitute proof of close relationship, as the features might have been preserved by chance in unrelated taxa. However, in this case, these genera are considered primitive and therefore primitive shared characters might be relevant.

Anginon and *Glia* seem to share a few similarities with *Bupleurum* (e.g. being glabrous), but unfortunately there are far more differences, like the heterophylly in *Anginon* (different types of leaves: juvenile and adult), and the fairly distinctive flowers and fruits. *Marlothiella* appears to be related to *Anginon*, but again it is very distinct from *Bupleurum*.

Considering that no clearly common morphological characters are shared between *Bupleurum* and these other woody umbellifers, finding the closest taxa of *Bupleurum* does not seem an easy task. It is possible, although less likely, that the closest relatives of *Bupleurum* are in genera containing only herbaceous plants, with their once woody taxa already extinct.

The herbaceous genus *Smyrnium* L. has been occasionally associated with *Bupleurum* (e.g. Leonardis *et al.*, 1997), however this genus only shows superficial similarities: e.g. *S. perfoliatum* L. might resemble *B. lancifolium* Hornem. or *B. rotundifolium* L., but, among many other differences, it does not have 'perfoliate' leaves like the name suggests, but only cordate-amplexicaul upper leaves, with the basal lobes sometimes superposing. Leonardis *et al.* (1997) show that the pollen of *Bupleurum* and *Smyrnium* are similar and of a primitive type. But, as already mentioned, sharing a primitive character is not necessarily a sign of close relationship.

At present, several molecular studies are being carried out in many genera of the family [e.g. Downie *et al.* (1996, 1998 & 2000); Downie & Katz-Downie (1996 & 2000 - *in press*); Katz-Downie *et al.* (1999); Plunkett *et al.* (1996a,b & 1997); and Valiejo-Roman *et al.* (1998)] and they might provide the extra information needed to clarify relationships.

Table 2.1 - Taxonomic History of the genus *Bupleurum*.

For convenience, *Bupleurum* at generic level has been omitted in the table in Grenier & Godron's classification (Gr. & Godr., 1848) and onwards.

Sprengel (1813b)	Hoffmann (1816)	Dumortier (1827)	Reichenb. (1828)	Gr. & Godr. (1848)	Boissier (1872)	Briquet (1897)	Drude (1898)	Calestani (1905)	H. Wolff (1910)	Koso-Polj. (1913)	Tutin (1968)	Cauwet (1976)
genus ***Bupleurum*** p.p.	genus ***Bupleurum*** p.p.	genus ***Diatropa*** p.p.	genus ***Bupleurum*** p.p.	section ***Perfoliata*** p.p.	section ***Perfoliata*** p.p.	section ***Perfoliata*** subsection ***Laevia*** (& subsect. *Lophocarpa* 1898)	section ***Perfoliata*** p.p.	section ***Perfoliata***	section ***Perfoliata*** subsections ***Laevia* & *Lophocarpa***	subgenus ***Diatropa*** section ***Laevia*** subsections ***Alophia* & *Lophocarpa***	section ***Bupleurum*** p.p.	subgenus ***Bupleurum*** section ***Bupleurum*** subsection ***Laevia***
_____ (genus *Bupleurum* p.p. - 1818)	_____	genus ***Diatropa*** p.p.	genus ***Trachypleu-rum*** p.p. (genus *Bupleurum* 'group' *Trachypleu-rum* -1830/2)	section ***Perfoliata*** p.p.	section ***Perfoliata*** p.p.	section ***Perfoliata*** subsection ***Rugosa***	section ***Perfoliata*** p.p.	genus ***Trachypleu-rum***	section ***Perfoliata*** subsection ***Rugosa***	subgenus ***Diatropa*** section ***Rugosa*** subsections ***Eurugosa* & *Granulata***	section ***Bupleurum*** p.p.	subgenus ***Bupleurum*** section ***Bupleurum*** subsection ***Rugosa***
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3. Review of the characters used in *Bupleurum*

3.1 Introduction

A natural classification of any group of organisms can only be achieved by combining data (characters) obtained from diverse areas of research. However, not all the information obtained may be useful to clarify the taxonomy of a particular group, and selection of characters is necessary. The taxonomic value of characters varies greatly between different groups, so it is only possible to know the usefulness of particular characters by studying them all.

As in all plant groups, morphology has been the principal source of taxonomic characters in *Bupleurum*. However, research has also been carried out in other areas, in particular in anatomy and karyology, but also in palynology, chemistry, and more recently in molecular biology. The following is a critical review of the characters that have been studied in *Bupleurum*, trying to point out the areas which seem more promising in the understanding of the relationships and evolution of the group.

3.2 Morphology

Bupleurum was early recognised as a distinct natural group mainly because of its simple and entire leaves that are almost unique in the *Umbelliferae* – see Taxonomic History (chapter 2). Other morphological features show the distinctness of the genus, particularly in that its plants are totally glabrous, and that most of its species have parallel-veined leaves (also extremely rare in the family). As the genus contains several woody species (woodiness is also very rare in the family), *Bupleurum* is generally regarded as a primitive (basal) group in the family.

If the genus is easily delimited (in total contrast to most genera in the family), its infrageneric classification and the delimitation of many of its taxa have for long been problematic.

Leaf shape, insertion and texture, and also habit, were the first characters used to distinguish different species of *Bupleurum* (e.g. Bauhin, 1623; Morison, 1672; Ray, 1686; Tournefort, 1694; and Linnaeus, 1753). The first formal

subdivision of the genus (Grenier & Godron, 1848) was based on leaf and bract characters (insertion of leaves, venation type and vein number; and absence/ presence and direction of bracts). Fruit morphological characters were also used in early studies, especially to distinguish *Bupleurum* from other genera, but have had a smaller contribution on the infrageneric classification.

Wolff (1910), in his comprehensive account of the genus, followed early authors, and mainly used leaf, bract and bracteole characters in his classification of the genus (he also used habit and fruit characters). Also, for the first time, Wolff used the morphology of the petal to distinguish species in *Bupleurum*. At present, the most generally accepted classification (Tutin, 1968) basically follows that of Wolff, and therefore leaf and leaf-like structures (bracts & bracteoles) still determine much of the classification of the genus.

Cerceau-Larrival (1962) carried out the first comparative study of seedling morphology in the *Umbelliferae*, a work which included 17 species of *Bupleurum* (16 species in the present concept). All the *Bupleurum* species studied had 1-veined, linear cotyledons, adding more evidence to the view of the genus as a natural group (see chapter 5 for discussion on seedling morphology).

Macroscopic characters have been largely used in *Bupleurum*, but there has been little research on micromorphology (restricted to pollen). The use of the SEM may provide some useful microscopic characters, namely from the study of the epidermal surface of different organs (leaves, stems, fruits).

See chapter 6 (section 6.2) for detailed discussion of morphological characters and character states used in *Bupleurum*.

3.3 Anatomy

Briquet (1897) used for the first time a combination of morphological and anatomical characters (stems, leaves and fruits) in the classification of *Bupleurum* (Alpine species). Together with the morphological description, he presented a detailed anatomical description for each species, and also, 2 keys to identification, one for morphological and the other for anatomical characters. Panelatti (1959), in her anatomical study of Moroccan species, and Cauwet (1976), in her PhD thesis on the genus, added information about other species. Arenas & García (1993) presented

a detailed study of the fruit anatomy of Iberian and Balearic *Umbelliferae*, subfamily *Apioideae*, including *Bupleurum* – see chapter 7 for detailed discussion of fruit anatomical characters.

The following is a list of stem and leaf anatomical characters that have been used in *Bupleurum*:

Stem:

- *ridges*: number, prominence.
- *collenchyma* pattern distribution.
- *sclerenchyma* pattern distribution (fibers/ sclereids in the cortex or associated to vascular bundles).
- *pith*: relative size (relation with total diameter), presence/ absence of secretory canals.

Leaf:

- *Epidermis*: cell shape and size, thickness of external cell wall, thickness of cuticle, stomata location (sunk/superficial).
- *Mesophyll*: structure (centric/ subcentric/ bifacial), secretory canals distribution.
- *Vascular bundles*: number, relative size.

Most of the characters/character states mentioned above have been used to describe and distinguish particular taxa, but there has been little discussion on their possible use in classification.

Briquet (1897) gave particular relevance to fruit characters, especially *vittae* (secretory canals – see chapter 7), to distinguish species, but he also used stem and leaf characters. Panelatti (1959) considered that the "secretory canals [of leaf, stem, and fruit] play an essential role in the anatomy of the genus", because "they allow us to establish relationships between species morphologically close, and moreover, to distinguish them from each other". So it seems that secretory canals are a good source of diagnostic characters, but if they are relevant to the classification is not yet clear. In Panelatti's opinion, the [many] other characters are less important, even when providing interesting information that complement the identification of species and their varieties. Nevertheless, the number of species studied by Panelatti was

small, and they were not even a representative sample of the only section included, Sect. *Isophyllum* (in the currently most accepted classification). Much research is still needed to understand the importance of the different anatomical characters in the taxonomy of the genus.

Wood anatomy

The study of the anatomy of the wood in *Bupleurum* is another possible source of characters, even if limited to the woody species or those that develop secondary xylem/phloem (perennial herbs with woody stock).

Rodriguez (1957, 1971) studied the anatomy of *Myrrhidendron* Coult. & Rose and other woody *Umbellales* (*Umbelliferae* and *Araliaceae*), and included one species of *Bupleurum*: *B. fruticosum* L. In his studies, he discussed a range of anatomical characters, giving special relevance to the wood. According to Rodriguez (1957), *B. fruticosum* is probably a primitive species in the genus because of its woody stem, but that some characters of its wood (short vessel elements, simple perforation plates, and 'tertiary' helical thickenings) suggest a fairly advanced condition. These 'tertiary' thickenings of the vessel elements (see Fig. 58F – Rodriguez, 1957) seem to be extremely rare in the family; they were also found in *Heteromorpha* Cham. & Schltdl., a possibly allied genus from C & S Africa, Madagascar and Yemen. Carlquist (1988) stated that the term 'tertiary' is incorrect, because ultrastructural studies had shown that the thickenings do not constitute an additional layer, but are part of the secondary wall. This particular "sculpturing" (Carlquist preferred this term) of the vessel walls has no easy interpretation as the feature seems to have arisen independently in different groups (in many families), both primitive and advanced (Carlquist, 1961, 1988). In any case, this feature seems to be useful for wood identification.

Schweingruber (1990) studied the wood anatomy of 4 species of *Bupleurum*: *B. foliosum* Salzm. ex DC., *B. fruticosum*, *B. fruticescens* L. and *B. spinosum* Gouan. No differences were found between the wood of *B. foliosum* and *B. fruticosum*, which supports the close relationship of these two species (mainly based on habit and leaf morphological characters). However, the wood of these two species can be distinguished from the other two taxa studied, *B. fruticescens* and *B. spinosum*, because the latter have: shorter rays, with larger number of cells; heterogeneous

uniseriate rays (rays are all homogeneous in *B. fruticosum* and *B. foliosum*), and smaller ray-vessel pits. Although not mentioned by Schweingruber (1990), there appear to be no differences between the sculpturing of the vessels elements of *B. fruticescens* and *B. spinosum* and those of *B. fruticosum* (see figs, p. 728 & 730 – Schweingruber, 1990). So, it will be interesting to investigate the distribution of this helical sculpturing on vessel walls in the woody species of the genus. These data suggest that wood anatomy may provide useful data in the study of relationships of the woody species of *Bupleurum*.

The stomata

All the stomata types that have been recorded in the *Umbelliferae* have been observed in *Bupleurum*: anomocytic, anisocytic, hemiparacytic, diacytic and paracytic. The classification of these types of stomata is based on shapes and arrangement of subsidiary cells. Stomata are also classified into perigenous and mesogenous, considering their development (ontogeny); both types have been recorded in *Bupleurum* (Gupta *et al.*, 1965; Guyot 1966, 1971, 1978; Ostroumova & Kljuykov, 1991) – see Metcalfe & Chalk (1979, p. 99-103) for explanation of the terminology.

Gupta *et al.* (1965) found that different organs of a single plant can have different types of stomata; e.g. in *Bupleurum tenue* Buch.-Ham. ex D.Don the stomata in the stylopodium are of anomocytic type, while in the rest of the organs they are anisocytic. Not only have different type of stomata been recorded in the same plant, but also the stomata ontogeny may differ between different organs. Although models of phylogeny of stomata have been proposed (e.g. Guyot, 1966, 1978), there is no consensus concerning stomatal evolution (Metcalfe & Chalk, 1979). Considering that different stomata type may occurr in the same plant and that there is no certainty concerning the phylogenetic relationships between the various stomata types, there seem to be very limited taxonomic use in the study of the stomata.

3.4 Pollen morphology

Pollen of several species of *Bupleurum* have been studied by Cerceau-Larrival (1955, 1959, 1962, 1971), Cerceau-Larrival & Roland-Heydacker (1978), Punt (1984), Merrgen (1994), and Leonardis *et al.* (1997).

Punt (1984) expressed some disagreement with the importance given by Cerceau-Larrival (1959, 1962) to certain characters, namely the P/E ratio (P = polar axis length; E = equatorial diameter), which she used as the main characteristic for identification of pollen. Punt found that most *Umbelliferae* species show large variation in the P/E ratio and, therefore, did not use this character in the key to identification. Cerceau-Larrival considered that the inner contour of the pollen is a more reliable character than the outer contour. Punt, however, preferred to use the outer contour of the pollen for 'practical' and 'technical' reasons; no further explanation was given, but one reason may have been that the SEM does not provide a view of the inner contour of the pollen. Nevertheless, both authors seem to agree in the general morphology of the pollen in *Bupleurum*.

Cerceau-Larrival (1962, 1971) considered the pollen of *Bupleurum* to be of a primitive type and described it as follows:

a) *type sub-rhomboidal* [according to the inner contour of the endexine, but external outline has a similar shape in *Bupleurum*].

b) *P/E ratio* = 1-1.5 [pollen can be ± isodiametric, but often polar axis is longer than equatorial diameter].

c) *symmetry* of order 3 [because it has 3 apertures or *colpi* (sing. = *colpus*) – there are rarely 2, 4 or 5 apertures in the pollen of umbellifers].

d) rectangular and prominent *endoaperture* with curved corners.

e) long *ectoaperture.*

f) thin *exine* (1-2 μm).

g) thickness of the exine is approximately the same around the pollen grain.

h) polar view sub-circular.

i) small size (P = 15-30 μm).

Punt (1984) remarked that the pollen of *Bupleurum* is easily distinguished by its rhombic or elliptic-rhombic outline in colpus view (position where the 'inner contour' of Cerceau-Larrival can be better seen), its columellae that are at the poles

(slightly or distinctly) longer than at the equator, its broad and often rectangular endoaperture, and the small size of the pollen; he indicated values of P/E ratio between 1.17 and 1.66 (P = 18-30 μm; E = 13-22 μm). Punt (1984) classified the pollen of the species he studied as the "*Bupleurum falcatum* type". Pollen of W Iranian species of *Bupleurum* was studied by Van Zeist in 1977 (referred by Punt, 1984), and according to Punt, they show a type of pollen similar to the European species.

There seems to be only minor variation in outlines, size and ornamentation between different species of *Bupleurum*. Cerceau-Larrival (1959) only gave a range of non-discriminatory measurements in her key to 'identification'. Punt (1984) only distinguished in his key, 2 of the 6 European species he studied.

The pollen in *Bupleurum* has been generally characterised as sub-rhomboidal. However, Merrgen (1994) in his study of 7 Chinese species, indicated two other types of pollen: suboval (one of the samples of *B. sibiricum* Vest ex Roem. & Schult.; the other two were typically sub-rhomboidal), and subrectangular (both samples studied of *B. chinense* DC.). The pollen of the latter seems so different from what has been recorded in the genus, that confirmation is needed to exclude any possibility of contamination or mixing of samples (it seems remarkably similar to the pollen of very distinct genera). If this type of pollen is confirmed for *Bupleurum*, it will cast doubts on the reliability of using pollen shape to distinguish genera in the family, especially if only the SEM is used.

There are other genera that have a similar sub-rhomboidal type of pollen, such as *Azorella* Lam. (S America), *Dethawia* Endl. (Pyrenees), *Hydrocotyle* L. (cosmopolitan), *Molopospermum* Koch. (W Mediterranean), *Physospermum* Cusson ex Juss. (Eurasia), *Pleurospermum* Hoffm. (Eurasia), and *Smyrnium* L. (Mediterranean) – [Cerceau-Larrival, 1962; Punt, 1984; & Leonardis *et al.*, 1997]. The differences are small, but apparently these genera can be distinguished from *Bupleurum* by their P/E ratio, shape and prominence of the endoaperture and/or details of the ornamentation (see Cerceau-Larrival, 1962, p. 103-104; Punt, 1984).

3.5 Karyology

Numerous chromosomes studies have been carried out in *Bupleurum*, in particular by Cauwet (1970, 1971, 1975a, 1976, 1979b); see also Cauwet *et al.* (1978). Cauwet (1979a) produced an account of the chromosome numbers known in the genus, and noted that she alone published counts for 76 taxa (including species, subspecies and varieties) out of the 90 taxa then known. Chromosome numbers for *Bupleurum* can also be found in Goldblatt (1981-1988), and Goldblatt & Johnson (1990-1998).

Five different basic (x) chromosome numbers have been indicated in *Bupleurum* (Cauwet, 1979a,b): x = 4, 6, 7, 8 and 11; the commonest being x = 8 [± 3/4 of total], followed by x = 7 [± 1/5]. Some species present various degrees of ploidy (polyploidy); e.g. *B. ranunculoides* L. have diploid (2n = 14 ⇒ 2x), triploid (2n = 21 ⇒ 3x), tetraploid (2n = 28 ⇒ 4x), and hexaploid (2n = 42 ⇒ 6x) populations (Küpfer, 1969; Cauwet, 1970, 1979b). Cases of aneuploidy and dysploidy have been recorded, especially in NW African taxa (Cauwet, 1979b) and in the Eurasian *B. falcatum* L. s.l. (Ohta, 1991). In the latter, the chromosome number variation is extraordinary: all numbers (2n) between 19 and 34 have been found, and there are also counts of 12, 16, 37 and 40 [*Aneuploidy* = occurrence of more or fewer chromosomes than an exact multiple of the haploid number. *Dysploidy* = occurrence of a series of basic numbers which usually differ by one chromosome (e.g. x = 5, 6, 7, 8, etc); contrarily to aneuploids, dysploid chromosome numbers are stable within a population].

Cauwet (1979c) also presented a cytophotometric study of nuclear DNA in 4 species of *Bupleurum*. In this study she verified that in *B. ranunculoides* the DNA content is directly proportional to the level of ploidy, suggesting that this is a case of autopolyploidy. Cauwet also compared the quantity of DNA between plants of different habit (perennial and annual herbs), and different basic number. She argued that of the 4 species studied, *B. junceum* L. (= *B. praealtum* L.), an annual herb, is the most evolved, because its quantity of DNA is lower than the perennials with the same basic number, and that *B. rigidum* is the most primitive because it has a higher DNA content; this in the highly arguable assumption that there must a reduction of DNA with the evolution of the species.

Cauwet (1976, 1979b,c) gave much importance to basic chromosome numbers in her interpretation of the evolution of *Bupleurum*. But, she also discussed cytological processes (aneuploidy and dysploidy) that can alter chromosome number and suggest an incorrect (original) basic number. The case of *B. rigidum* (see Cauwet, 1979b, p. 58-59) is paradigmatic. Cauwet assumed that the basic number for *B. rigidum* was x = 8, with the species being diploid (2n = 16). However, when counts of 2n = 14 were found for the species, she interpreted the situation as a case of dysploidy, with change from 8→7; but there is no reason why it could not be the other way round.

Chromosome numbers alone have very limited taxonomic value. However, the study of chromosome morphology can provide extremely valuable data in taxonomic and phylogenetic studies (Jong, 1997).

So far chromosome morphology has been only studied in Asian populations (Russia, China & Japan) of *B. falcatum* s.l. (including taxa such as *B. chinense* DC., *B. komarovianum* O.A.Lincz., and *B. scorzonerifolium* Willd.), *B. longiradiatum* Turcz., *B. sachalinense* F.Schmidt, and *B. angustissimum* (Franch.) Kitagawa – Gorovoy *et al.* (1980); Gorovoy & Volkova (1987); Hore (1979); Jiang *et al.* (1994); Li *et al.* (1994); Ohta, (1991); and Ze-Hui *et al.* (1995).

The following are some *morphological characters* that can aid the identification of individual chromosomes (Jong, 1997):

- *Chromosome shape*: (metacentric, submetacentric, acrocentric, telocentric).
  a) arm ratio = shorter arm/ longer arm, or longer arm/ shorter arm;
  b) centromeric index (or F%) = length of shorter arm/ total length of chromosome.
- *Chromosome size.*
- *Satellites*: position, shape, number and size.
- *Heterochromatin* and *banding pattern.*

Other source of information are the B-chromosomes (supernumerary chromosomes, normally smaller and variable in number) that are present in many species of plants. Their role is not clear, but they may be relevant in the evolution of populations. B-chromosomes have been found in *B. bicaule* Helm, *B. chinense*, *B. falcatum*, *B. mesatlanticum* Litard. & Maire, *B. ranunculoides* and *B. rigidum* L.

(Cauwet, 1979a,b; Goldblatt, 1981-1988; Goldblatt & Johnson, 1990-1998; and Ohta, 1991).

After identification of chromosomes, it is possible to compare karyotypes of possibly related taxa, or individuals of a single taxon that show variation in chromosome number. This analyses can provide evidence on how chromosome numbers have changed and the mechanisms involved (polyploidy, fusion or fission of chromosomes, etc.) – see e.g. Ohta, 1991.

3.6 Chemistry

Research in the distribution of particular chemicals, especially secondary metabolites, such as coumarins, flavonoids, acetylenic compounds, triterpenes, saponins and seed oils, have provide interesting data in the taxonomy of the *Umbelliferae*, in particular at the generic and suprageneric level, but also concerning relationships between families.

Some of these chemosystematic studies included species of *Bupleurum* (Bohlmann, 1971; Crowden *et al.*, 1969; Harborne, 1971; Harborne & Williams, 1972; Hegnauer, 1971; Nielsen, 1971; Saleh *et al.*, 1983). Carbonnier & Cauwet (1979) presented a review of the chemical compounds known then for the genus. Some other chemical studies have provided scattered information on the chemistry of particular species of *Bupleurum* [e.g. González *et al.* (1990a,b); Luo *et al.* (1993a,b); & Pistelli *et al.* (1993)]. Unfortunately, only comparative studies can provide useful data in taxonomy.

Carbonnier & Cauwet (1981) developed the first comparative phytochemical study in *Bupleurum*. They studied 124 "populations" [individuals from different localities] of *Bupleurum* and other [possibly] related genera, and found 78 different chemical compounds. However, none of these "constituents" were identified, and band patterning in the chromatograms of the 33 'species' (42 taxa) of *Bupleurum* was simply compared. These authors argued that "the exact nature of a compound is of rather minor importance", and that "presence of a given substance should be regarded as a true systematic character, allowing relationships between species to be appraised". However, without being absolutely sure that bands in the same position in chromatograms of different taxa do indeed correspond to the same compound, the

data obtained cannot be reliably used in the taxonomy of the group. Furthermore, some of the chemical variation may be due to different environmental conditions. Both abiotic factors (e.g. UV light, temperature, mechanical damage), and biotic stress factors (e.g. fungal infection, insect feeding), can induce biosynthesis and accumulation of particular substances, such is the case with furanocoumarins (Berenbaum, 1981, 1990). Therefore, absence of a compound is not evidence enough that such cannot be produced, as it was assumed by Carbonnier & Cauwet (1981).

3.7 Molecular biology

In recent years, molecular studies have provided very valuable data in the study of plant phylogeny. See chapter 9 (section 9.1) for discussion on the different approaches and methodologies in molecular systematics.

Bupleurum species have been included in some of the molecular studies carried out in the *Umbelliferae*.

Sequences of **chloroplast** genes (*rbc*L, *rpo*C1 intron, and *mat*K) have been obtained for a few species of *Bupleurum*: *B. chinense* DC. (*rpo*C1 intron), *B. falcatum* L. (*rbc*L, *mat*K), *B. fruticosum* L. (*rbc*L), *B. longiradiatum* Turcz. (*rbc*L), *B. ranunculoides* L. (*rpo*C1 intron), and *B. scorzonerifolium* Willd. (*rbc*L) – Kondo *et al.* (1996); Plunkett *et al.* (1996a,b & 1997); and Downie *et al.* (1998). A species of *Bupleurum* (*B. chinense* 'Franch.') was also included in a recently published study of chloroplast restriction site data in the *Umbelliferae* (Plunkett & Downie, 1999). In the analysis of phylogenetic relationships, *Bupleurum* consistently appears as a basal clade within subfamily *Apioideae*.

Choi *et al.* (1996) obtained the first **ITS** sequences (internal transcribed spacers of nuclear ribosomal DNA) in *Bupleurum*: for *B. euphorbiodes* Nakai, *B. komarovianum* O.A.Lincz. (two samples, one from Japan and another from Korea), *B. longiradiatum*, and *B. scorzonerifolium*. These authors also carried out a RFLP analysis (Restriction Fragment Length Polymorphism) of the same samples sequenced for ITS, obtaining similar results in the relationships of the taxa.

ITS sequences have been also obtained for *B. falcatum* (Lee & Rasmussen, 1998; & Valiejo-Roman *et al.*, 1998). In the analysis of Lee & Rasmussen (1998), the 3 species of *Bupleurum* form a separated clade, but appear in a larger group

including genera such as *Aciphylla* J.R.Forst. & G.Forst., *Daucus* L., *Cuminum* L., *Laserpitium* L. and *Thapsia* L. This is definitely not in agreement with the chloroplast DNA (cpDNA) tree of *rpo*C1 intron (Downie *et al.*, 1998) as Lee & Rasmussen (1998) argued. These results are fairly surprising; unfortunately, the authors did not published the aligned sequences.

In contrast, the phylogenetic analysis of Valiejo-Roman *et al.* (1998), shows *B. falcatum* in a basal position within *Apioideae*, which is indeed in agreement with the results of cpDNA. These authors also noted that the sequence of *B. falcatum* is highly divergent and that it could not be aligned unambiguously with the rest of the taxa examined. Nevertheless, the number of *Bupleurum* taxa sequenced is still small and not at all representative of the variation in the genus.

3.8 Conclusions

All the research fields mentioned above are potentially relevant in the study of the taxonomy of any plant group. However, in the particular case of *Bupleurum* and in a project with limited time of research, some of these domains, for different reasons, do not seem immediate good sources of taxonomically useful characters, at least, at the generic level.

The study of stomata has not yet produced reliable information to be used in the taxonomy of the genus.

Considering the great uniformity of pollen grain morphology in *Bupleurum*, the interest of palynology seems to lie at suprageneric levels, and therefore of no immediate relevance to the present project.

Although numerous chromosome counts have been recorded in the genus, there is only a handful of studies which included chromosome morphology. This is an area of research with great taxonomic interest. However, karyological work requires living plants, either: **a)** flowering plants, for the study of haploid set of chromosomes, in developing pollen mother cells of young anthers, or in embryo-sac mother cells in ovules; or **b)** young plants, for the study of mitotic cells in active meristems, normally from root tips (see e.g. Jong, 1997). Unfortunately, living plants or seeds for germination are only available for a few species in the genus, and none

for the most problematic taxa in the area of study (mainly taxa endemic to NW Africa).

Several chemical studies have been carried out in *Bupleurum*. But there is no information on which chemical compounds could be taxonomically relevant in the genus. Also, chemical information, even from comparative studies, should be carefully interpreted, as uncontrolled environmental factors may affect chemical production.

Therefore, the areas that seem most promising in the study of the taxonomy of *Bupleurum* are: **a)** morphology (macroscopic and microscopic characters); **b)** anatomy; and **c)** molecular biology (DNA sequencing).

In any taxonomic study, morphology is undoubtedly the most immediate source of characters. A revision of the taxonomy of any plant group should always start with a review and assessment of the morphological characters that have been used to define the taxa and their classification. This is even more important if the group has not been recently revised, and even in this case such review(s) should be critically questioned. Morphological study does not involve expensive or complex methods, and can save a considerable amount of funds if expensive techniques are intended to be used (like those of molecular biology).

The use of anatomical data in the taxonomy of the genus has not yet been fully tested. But anatomy has often provided very valuable data to clarify relationships in plant groups.

The molecular study of *Bupleurum* is still at the very beginning, so it is not possible to know which methods will be more successful in clarifying relationships in the genus. DNA sequencing is probably the best strategy to study phylogenetic relationships (see section 9.1). Chloroplast sequences have been largely used to study generic and suprageneric relationships, but have had a more limited used at the infrageneric levels (Palmer, 1988; Olmstead & Palmer, 1994). More recently, nuclear ribosomal DNA, in particular the ITS region, has been proven very useful at the generic level (Baldwin, 1992; Baldwin *et al.*, 1995; and Hillis & Dixon, 1991). ITS region has also been successfully used in subfamily *Apioideae* (Downie & Katz-Downie, 1996). *Bupleurum* ITS sequences seem difficult to align with those of other

genera in the family (Valiejo-Roman *et al.*, 1998), but, on the other hand, were easily aligned together, and showed significant sequence divergence to study relationships within the genus.

Molecular study, in particular DNA sequencing, can provide a very abundant set of data that can elucidate phylogenetic relationships at all levels of the classification. DNA evolves in a more regular mode than morphological characters and therefore can provide a clearer view of relationships (Li, 1997). Potentially, molecular data can solve problems that could not be dealt with by traditional approaches.

Molecular data should be used as an important complement to other sources of data (morphological, anatomical, karyological, etc) and not as a substitute. Morphology and other traditional studies are extremely valuable, and their study over many decades (and centuries!) have created the foundation for more modern and sophisticated approaches.

For a more detailed discussion on the advantages and limitations of both morphological and molecular characters see Hillis (1987), Moritz & Hillis (1996), and Patterson *et al.* (1993).

4. Introduction to *Bupleurum* in the W Mediterranean and Macaronesia

4.1 Delimitation of the geographical area studied

Although the need for a global revision of the taxonomy of *Bupleurum* is a desideratum, research on diversity and a reassessment of taxa is still needed in many areas of the total range of the genus. One such area is the W Mediterranean, more precisely, NW Africa where a high number of poorly known species/taxa are found.

In the context of the present work the *W Mediterranean* includes:

1) *The Iberian Peninsula* (Portugal, Spain and Andorra), with the Pyrenees acting, at least in part, as a natural phytogeographic barrier that separates the area from the rest of Europe. All Pyrenean taxa of the genus have been dealt with, including a smaller number of specimens from the French side of the Pyrenees.

2) *The Balearic Islands*.

3) *NW Africa* (Morocco, Algeria and Tunisia), with the Sahara desert acting as the southern/eastern barrier limiting dispersal – the genus is not found S of the Sahara nor in C Africa.

Macaronesia was also included, because of its known affinities with both Iberian and African floras (Sunding, 1979; Bramwell, 1985). The biogeographical region of Macaronesia includes 5 Atlantic archipelagos: Azores, Madeira, Salvage Islands, Canary Islands and Cape Verde. *Bupleurum* is native only in Madeira and Canary Islands.

This study is mainly concerned with the *Bupleurum* taxa in this geographical area, but for a better understanding of the diversity of the taxa, a selection of material from throughout the whole range of distribution of the species was also consulted.

4.2 The species in the area: a preliminary overview

Most of the problematic *Bupleurum* taxa in the study area are, not surprisingly, N African, with several of its endemic taxa very poorly known. However, some problems, in particular concerning rank, were also found in the Iberian, Balearic and Macaronesian taxa. The following table summarizes the results of an initial evaluation of the taxa described dating from the start of my PhD studies. The vast majority of the taxa listed below still needed typification. Some of the herbaria where type material is found is indicated in the table, e.g.: (→ MPU) – see Appendix I for explanation of herbaria abbreviations. Author's names abbreviations follow Brummit & Powell (1992).

Table 4.1 - The *Bupleurum* species described in the W Mediterranean and Macaronesia: a preliminary overview. Afr. = Africa; Isl. = Islands; P. Iber. = Iberian Peninsula.

Taxa	World Distribution	Comments
B. acutifolium Boiss.	P. Iber.	Very restricted distribution. Morphologically close to *B. barceloi* (endemic to the Balearic Isl.) and African taxa such as *B. atlanticum* and *B. montanum*.
B. album Maire	Morocco	Very distinct species with several unique features in the genus, e.g. very compact inflorescence, 'white' flowers, petals with fimbriate inflexed apical lobe and very long styles.
B. angulosum L.	Pyrenees	Clearly distinct species, sometimes mistaken for its close relative *B. stellatum* (endemic to the European Alps), but easily distinguished by the bracteoles: they are connate (= fused) in the latter.
B. antonii Maire	NW Afr.	Doubtful taxon. No clearly distinctive features were given in the protologue; a likely synonym of a previously described taxon. Type material needs to be studied (→ P & MPU).

Taxa	World Distribution	Comments
B. atlanticum Murb.	NW Afr.	Problematic taxon. Characters used to distinguish this species show a high degree of plasticity. Cauwet & Carbonnier (1975, 1976, 1977) presented a detailed study (morphology, anatomy, phytochemistry, karyology) of this taxon. Despite not finding a single good character to identify the species, they proposed the subdivision of the species in 4 new subspecies: *atlanticum*, *aionense*, *algeriense* & *mairei* – unfortunately they are only distinguishable by chromosome number or geographical distribution!
B. balansae Boiss. & Reut.	NW Afr.	Because of its morphological affinities it has been considered a synonym or, more recently, a subspecies of *B. fruticescens* (Bolòs & Vigo, 1974). Requires reassessment of taxonomic status.
B. baldense Turra	Europe	Clearly a distinct species, but has been on occasions mistaken for very different species like *B. semicompositum*. Distintictive features need to be more clearly described in keys of identification.
B. barceloi Coss. ex Willk.	Balearic Isl.	Generally regarded as an endemic in the Balearic Islands, but more recently proposed as a subspecies of *B. dianthifolium*, an endemic of the island of Marettimo, near Sicily (Bolòs & Vigo, 1974). Requires reassessment of taxonomic status.
B. benoistii Litard. & Maire	Morocco	Distinct species, but can be easily mistaken for poorly developed specimens of other NW African taxa.
B. bourgaei Boiss. & Reut.	P. Iber.	No clear distinctive features. A likely synonym of *B. ranunculoides* as suggested by Tutin (1968). Type material needs to be studied (→ G).
B. canescens Schousb.	Morocco Canary Isl.?	Fairly distinct taxon, showing strong affinities to the Macaronesian endemic taxa. *B. canescens* is found in the area that is regarded, by some authors, as 'the Macaronesian enclave' in NW Africa (Sunding, 1979, p. 14-15).
B. chouletii Pomel	NW Afr.	Doubtful taxon. Quézel & Santa (1963) considered it to be a synonym of *B. oligactis* Boiss. – itself another problematic taxon.

Taxa	World Distribution	Comments
B. dumosum Coss. & Balansa	Morocco	Distinct species. A subshrub with apparent affinities to *B. fruticescens*, but with ± short herbaceous leaves appearing in 'bundles' (= clusters) – a very rare feature in the genus.
B. falcatum L.	Eurasia	A very polymorphic taxon. Two of its subspecies have been cited for the area under study (subsp. *falcatum* & *cernuum* – see Tutin, 1968). Difficult to distinguish from other Iberian species after withering of the basal leaves (e.g. *B. praealtum*).
B. faurelii Maire	NW Afr.	Doubtful taxon. No clearly distinctive features were provided in the protologue. A likely synonym of a previously described species (*B. montanum* or *B. atlanticum* ?). Type material needs to be studied (→ MPU).
B. foliosum Salzm. ex DC.	P. Iber. & Morocco	Clearly distinct species. Requires a better definition of distinctive features to avoid confusion with some material of *B. gibraltarium.*
B. fruticescens L.	P. Iber. & NW Afr.	There seems to be a continuous morphological variation between *B. fruticescens* and *B. spinosum* in the Iberian material. This supports the view of the two taxa as subspecies of *B. fruticescens* as proposed by Bolòs & Vigo (1974). However, there is no intermediate material between subsp. *spinosum* and the other subspecies proposed: subsp. *balansae* (NW Africa). Detailed revision of these taxa is necessary.
B. fruticosum L.	Mediterr.	Clearly distinct species. No special problems.
B. gerardii All.	Eurasia	Polymorphic taxon. Very often mistaken for *B. praealtum*: none of the characters used seem to be reliable to distinguish the two species.
B. gibraltarium Lam.	P. Iber. & NW Afr.	Clearly distinct species. No special problems.
B. handiense (Bolle) G. Kunkel	Canary Isl.	Doubtfully distinct from *B. canescens*. Originally published as *B. canescens* var. *handiense*.

Taxa	World Distribution	Comments
B. intermedium Poir. in Lam.	Eurasia	Generally regarded as a synonym of *B. lancifolium*, but treated as a different species by Snogerup (1972). However, the differences indicated (all in size) are minor, and there are no real gaps in the values, i.e. variation seems to be continuous.
B. lancifolium Hornem.	Eurasia N Afr. (Macaron.)	Polymorphic but distinct species; often mistaken for *B. rotundifolium*. Some authors (e.g. Snogerup, 1972; Jafri, 1985) preferred to classify part of the material of *B. lancifolium* in a different species (*B. intermedium* Poir. in Lam.; or *B. subovatum* Link ex Spreng.).
B. lateriflorum Coss. ex Wolff	Morocco	Distinct species. Shrub with very characteristic inflorescence: flowering stem with a terminal umbel, and several smaller and shortly pedunculate lateral umbels (one umbel per upper node).
B. mauritanicum Batt.	NW Afr.	Doubtful taxon. No clearly distinctive features were given in the protologue. Quézel & Santa (1963) suggested that it might be a synonym of *B. oligactis* (another problematic taxon). Type material needs to be revised (→ P).
B. melillense Pau	NW Afr.	A synonym of *B. balansae*. Holotype (MA 86591!) studied during visit to Real Jardín Botánico de Madrid (1994).
B. mesatlanticum Litard. & Maire	NW Afr.	Doubtful taxon. No clearly distinctive features were provided in the protologue. A likely synonym of a previously described species (*B. atlanticum* or *B. montanum*?). Type material needs to be revised (→ MPU).
B. montanum Coss.	NW Afr.	Polymorphic species. Not always easily distinguished from *B. atlanticum*.
B. odontites L.	Europe & N Afr.	Clearly distinct species, but often mistaken with *B. baldense*. *B. fontanessi* Guss. (used by Tutin, 1968) is a later name for this species.
B. oligactis Boiss.	NW Afr.	Problematic taxon. No clearly distinctive features were given in the protologue. Type material needs to be studied (→ G).

Taxa	World Distribution	Comments
B. plantagineum Desf.	Algeria	Fairly distinct species, apparently with a very restricted distribution.
B. praealtum L.	Europe	Polymorphic taxon, often confused with *B. gerardii* (see above).
B. procumbens Desf.	NW Afr.	Regarded as a synonym of *B. tenuissimum* in recent treatments.
B. ranunculoides L.	Europe	Species very variable in size and in width of leaves and bracteoles. Two subspecies have been indicated for the Iberian peninsula (see Tutin, 1968). See also notes on *B. bourgaei* above.
B. rigescens Maire & Sennen	NW Afr.	A synonym of *B. balansae*.
B. rigidum L.	P. Iber. NW Afr.	Clearly distinct species, easily recognizable by its very hard leaves with very prominent veins. The basal leaves of subsp. *rigidum* and subsp. *paniculatum* can be very different in shape and width, but intermediate specimens are readily found.
B. rotundifolium L.	Eurasia	Clearly distinct species. No special problems.
B. salicifolium R.Br. ex Buch	Madeira Canary Isl.	This species has been subdivided into two subspecies (Cauwet & Sunding, 1981): subsp. *salicifolium* and subsp. *aciphyllum*. However the only difference between the subspecies is the width of the leaves.
B. semicompositum L.	Eurasia, NAfr. Macaron.	Clearly distinct species. Very characteristic fruits: papillose and with no visible ridges.
B. spinosum Gouan	P. Iber. NW Afr.	See notes of *B. fruticescens* above.
B. subspinosum Maire & Weiller	Morocco	Apparently clearly distinguishable by its "simple umbels". Seems to be close to *B. spinosum*. No material was initially seen. Type material needs to be revised (→ MPU).
B. tenuissimum L.	Eurasia N Afr.	Clearly distinct species. It can be mistaken for *B. semicompositum*, but easily distinguished by the different ornamentation of the fruits.

5. Cultivation

5.1 Introduction

The study of living plants is always an important complement to data obtained from herbarium specimens. However, the study of plants in the wild is not always possible, and therefore *ex situ* cultivation is often required. Even when study in the wild is possible, cultivation provides additional data, in particular about seedlings and further stages of development that are difficult to observe in the wild. Also, the degree of phenotypic variation (i.e. variation that results from different environmental conditions) can be better estimated in plants under cultivation. Furthermore, cultivation can be of vital importance to preserve and propagate rare and endangered taxa.

The main aims of the cultivation work carried out were: **a)** to obtain living plants from *Bupleurum* species/taxa available in seed collections or from fruits collected by myself; **b)** to study the morphology of living plants, especially seedlings; and **c)** to enlarge the number of species under cultivation in botanical gardens, in particular, in the Jardim Botânico de Coimbra (Portugal) and the Royal Botanic Garden Edinburgh (RBGE).

5.2 Material & methods

Fruits were requested and received from 34 Botanic Gardens. Unfortunately, fruits are only available for a reduced number of species of *Bupleurum*, and generally, fruits cannot be obtained for the taxa that most need taxonomic revision, specially from N Africa. A few of the samples used for cultivation were collected by myself in the wild (Portugal).

Eighteen species of *Bupleurum* (34 accessions) were sown and cultivated in the greenhouses of ICMB (Institute of Cell and Molecular Biology, Edinburgh University).

After a cultivation experiment in Portugal (Departamento de Botânica, Universidade de Coimbra) with pots placed in the open air, a similar procedure was

used in the first sowing in Edinburgh, but with pots placed in greenhouses.

The first sowing in Edinburgh was in May 1995. This is a late sowing time, but not too late if we take in account that: (a) the material was cultivated indoors, and (b) the late flowering times in places of high latitude. An earlier sowing was not possible as I started my work in Edinburgh in April of that year.

Pots were prepared with standard compost (Professional Levington Compost F2 – without sand: fine structure, medium nutrient, neutral pH). Mericarps were scattered evenly on the soil surface and covered with a thin layer of soil – only enough to cover the seeds. Amount of mericarps sown per pot depended mainly on the number available per accession (for some only a few fruits were received).

After failure of germination in several species (as had happened before in Coimbra), some modifications were carried out for a second sowing in July-August 1995. This time pots were filled with a mixture of the standard compost with peat and sand (ca. 3:1:1), to avoid soil compactness. Mericarps were sown as before, but covered in two different ways: a) a fine layer of soil, with the pot then covered with translucent plastic; and b) a fine layer of coarse grit uncovered. Both procedures help to preserve the humidity of the soil, and 'b)' also avoids fungal development on the soil surface.

In some pots where the germination was successful, it was necessary to transplant the seedlings into different pots to let the plants develop more freely. Seedlings (4-8 cm high) should be moved with the surrounding soil to minimize disturbance of the roots. However, when the pots are overcrowded, separation of single seedlings with no damage is not possible. In this case, groups of seedlings should be transplanted together to the new pots.

When the seedlings produced the first leaves a herbarium specimen was collected for each of the species – these specimens also show the cotyledons as first true leaves are produced shortly after germination. Fruits and vouchers specimens were also collected from all flowering and fruiting species. Voucher specimens were not collected when just one seedling was produced or when all seedlings died shortly after germination.

The data registered in the next section refers to both Coimbra and Edinburgh experiments of cultivation.

5.3 Results & discussion

Germination data, including the species names, accession quantities, rate and speed of germination, and morphological characters of the seedlings are presented in Table 5.1. This table summarizes data obtained from both Edinburgh and Coimbra cultivated material, including all the species sown in Edinburgh, but does not include some of the species that were just sown in Coimbra and did not germinate – fruit material is often wrongly identified or is a mixture of different species, and, for most of the taxa, accurate identification is only possible after germination or usually much later.

A) Germination data

Germination occurred in 11 of the 18 species sown in Edinburgh (1995); in the previous year the rate was 13 out of 21 (Coimbra). Most of the species are slow to germinate, more than 1 month is needed, but annuals usually take a shorter time, normally c. 2 weeks.

Germination of the fruits of a single sample seems to occur ± simultaneously. In several cases, the same happened in different samples of a species (e.g. *B. rotundifolium*, *B. gerardii* and *B. fruticosum*), but, normally a few of the fruits take a further time to germinate (up to 2 months later). One reason for this is that even seeds produced by a single plant might differ in their degree of dormancy. Seeds might receive a different hormonal supply depending on the position in the mother plant or the time that they are produced (Bewley & Black, 1994, p. 222). Another reason for the staggered germination could be the mixed nature of the sample: collected either from different plants in the area of collection, or from plants of different populations; dormancy often varies with provenance due to different environmental conditions (Baskin & Baskin, 1998, chapter 8). In any case, germination that is spread over the time has great adaptative value, especially in habitats with frequent environmental fluctuations. If early germinating seedlings are killed, a second or a third batch might be more successful (Baskin & Baskin, 1998, chapter 12).

Germination completely failed in some species (*B. angulosum, B. stellatum* and *B. gibraltarium*). Also, in several others (e.g. *B. lancifolium* and *B. falcatum*) only 1-4 seedlings were obtained (from 20 to much more than 100 fruits sown). In the case of *B. falcatum* the poor germination might be explained because of the short viability of its seeds. According to Wigginton (1999), the seeds of this species are viable only for about a year, and basically all the material I have sown was older than that. As a curiosity, the only accession that germinated (Acc. No 118 – see Fig. 5.3B) produced a few plants that do not correspond to the typical material of the species (e.g. the one found in the Iberian Peninsula and in Britain). Although, origin of this sample is not known, it is likely to be from a taxon of the problematic eastern group (E Europe and Asia) of *B. falcatum* s.l. – a plant from this accession is growing at the Royal Botanic Garden Edinburgh (Alpine Yard).

In total contrast, one species, *B. rotundifolium*, shows an extraordinary rate of germination. A large number of seedlings were obtained for all the accession numbers received (9 accessions from different origins). Seedlings developed easily in mature plants and produced abundant fruits (see Fig. 5.3C). Such was the amount of fruits, that it became impossible to avoid contamination of the surrounding pots. Nevertheless, this overproduction of seedlings and fruits only seems to occur indoors, as the species is fairly rare in the wild, in spite of having a fairly broad distribution in Europe and W Asia. Baskin & Baskin (1998, p. 374-380) have studied the germination of this species and found that their seeds have non-deep dormancy, easily broken by warm temperatures, and that percentage of germination is higher in darkness than in light.

Bupleurum rotundifolium is definitely very easy to cultivate and reproduce. So it is not surprising that this is the only species of *Bupleurum* that seems to be commercially cultivated, although in a reduced scale – I have seen it on sale in flower shops and supermarkets in Britain (common name: thorow-wax). The flowering branches of *B. rotundifolium* are used for home decoration – this species also meets the requirement of being 'pretty' or maybe just interesting!

Despite the last example, the rate of germination for most of the *Bupleurum* fruit samples was low. Possible explanations for these results are:

1) Fruits were not ripe or properly developed when collected.

2) Fruits were too old.

3) Conditions of cultivation were not appropriate – i.e. one or any combination of several factors (soil pH, soil porosity, humidity, temperature, number of hours of daylight) was unsuitable for a particular species under cultivation.

4) Techniques used were inappropriate.

Conditions of cultivation are very difficult to assess, as what is optimal for one species might be completely inadequate for another. In general, cultivation in greenhouses offers a very unnatural environment, but sometimes the difference is for the better – e.g. water supply is more reliable. In my experience with *Bupleurum*, germination did not seem to be affected by conditions other than proper humidity, warm temperature and enough time allowed to break fruit dormancy.

However, conditions of cultivation are indeed crucial for the proper development of seedlings into fully mature plants. For example, several seedlings were obtained from *Bupleurum rigidum* subsp. *paniculatum* (Acc. No 89), but the plants developed poorly. Two of the plants flowered, but fruits never developed, and eventually they all died. This is not surprising as the conditions in the greenhouses hardly matched the natural environment of *B. rigidum*: a plant of dry areas in the Iberian Peninsula and NW Africa, which, as many umbellifers, flowers in the height of the summer.

Concerning procedures, there was no significant difference in the rate of germination between the first sowing and the second sowing where a few modifications were introduced. However, it seems that covering the soil with a thin layer of coarse grit, does indeed help to the further development of the seedlings – soil water is retained longer and invasive plants or fungi do not develop so easily. Covering the pots with translucent plastic helps to retain soil humidity, but the beneficial effect on the earlier stages of germination was not obvious, it might be easily seen in pots placed outdoors under dry weather conditions.

I have successfully germinated all fruit material collected by myself, either from the wild or cultivation. Although in a few cases I only obtained a small number of seedlings. Later I discovered that even some of the fruits (mericarps) that I had collected believing as fully ripe, actually lacked endosperm (i.e. they were hollow), and so would never germinate. This problem can be partially avoided if the quality of

the fruits is assessed before collecting; this is even more important when collecting in the wild. We can do this by cutting a few of the fruits in half to see if the endosperm is present – if the growing conditions have not been ideal in that particular season, many of the fruits will lack endosperm. When the amount of fruit is limited, we could try our luck, but in these case we should not collect at all from wild material for conservation reasons.

For some of the samples the actual date of collection was indicated, varying between 1 to 5 years old; for others the only reference was the year of the *Index Seminum* of the sample requested, with date of collection not being referred. Other samples that did not germinate had been collected recently. So, although the date of collection could be crucial, this factor by itself does not explain the failure of germination for recently collected material. Other factors that affect the quality of the seeds are the conditions of storage (temperature, humidity).

As mentioned before, the likely reason for the failure of germination of seeds of *B. falcatum* was that they were too old. However, I have successfully germinated material that was at least 5 years old from other species (e.g. *B. gerardii*, an annual herb, and *B. fruticosum*, a shrub). Therefore, the short viability registered in *B. falcatum* (Wigginton, 1999) is not necessarily a characteristic of other species in the genus. Also, research is still needed to confirm that other populations show the same short viability of *B. falcatum* in Britain – the species is here critically endangered, but not in the rest of Europe.

B) Seedling morphology

Considering the seedling characters in *Bupleurum*, all material have 1-veined linear cotyledons (see Fig. 5.1); this is a generic characteristic (Cerceau-Larrival, 1962). However, length and colour vary between different species; hypocotyls also have some variation in length and colour (see Table 5.1).

Identification of species from seedlings with only cotyledons is normally not possible. However, I have found one species, *B. rigidum*, that can be easily identified at this early stage. Seedlings of *B. rigidum* have an extremely short hypocotyl (c. 1 mm) and considerably long (3-5 cm) and narrow (0.5-1 mm) cotyledons – cotyledons in *Bupleurum* are usually less than 3 cm long and 1-2 mm broad. There seems to be a

correlation between the very long cotyledons of *B. rigidum* and its also very long adult leaves (up to 45 cm) – to my knowledge the longest in the genus.

First true leaves show more significant variation, specially in shape and venation type – see Figs 5.2-5.3 (A,B,D). They are normally similar to the following leaves and therefore useful for identification (leaf characters, in particular venation type, define the major groups – sections and subsections – in *Bupleurum*).

Despite the fact that most of species can only be correctly identified from flowering and fruiting material, it is possible to recognise the group from which they belong from the seedlings that have already produced first leaves. These groups are in some cases larger than the currently accepted sections. For instance, *B. rotundifolium, B. lancifolium* [Sect. *Bupleurum/ Perfoliata*] and *B. longifolium* [Sect. *Diaphyllum*] have very similar seedlings. Other morphological characters support a closer relationship between the plants of these 2 sections, namely insertion and veining of the leaves and also the morphology of the inflorescence. Therefore, morphology of first leaves may provide some useful data about relationships of the major groups.

Although the colour of seedlings might be useful to distinguish some of the species, it is not a reliable character, and is open to subjectivity unless colour charts are used. Colour varies in the same species under different conditions of cultivation, in particular, seedlings become reddish or purplish under intense light radiation – the same happens in later stages of development.

Cerceau-Larrival in her PhD thesis on seedlings and pollen of *Umbelliferae* (1962) described the following species of *Bupleurum* (5 of the 17 she studied) as having hairy cotyledons: *B. rotundifolium*, *B. gerardii*, *B. praealtum*, *B. falcatum* and *B. canescens*. I have already successfully germinated *B. rotundifolium* (9 different accessions), *B. gerardii* (5 acc.) and *B. praealtum* (3 acc.) from rather different origins, and I germinated an accession of *B. handiense*, a taxon that I regard as a synonym of *B. canescens* (see chapter 10). I also obtained a few seedlings of *B. falcatum* (most of the accessions failed to germinate). Yet despite careful observation, I have not confirmed the recorded hairness. All material cultivated so far produced seedlings, and therefore cotyledons, that are totally glabrous.

At the present time some of the *Bupleurum* material cultivated by myself has been transplanted into the main garden in both the Jardim Botânico de Coimbra and in the Royal Botanic Garden Edinburgh (RBGE).

Previously, there were no *Bupleurum* plants growing in Coimbra, but now a few plants of 4 shrubby species have been successfully transplanted: *B. fruticescens* subsp. *spinosum* (Acc. No 34 – see fig. 5.3E), *B. fruticosum* (Acc. No 5 & 15), *B. canescens* 'var. *handiense*' [= *B. handiense*] (Acc. No 28), and *B. salicifolium* (Acc. No 29) – the last two taxa are endemic to Macaronesia, and '*B. handiense*' has a very restricted distribution in the Canary Islands.

Five species were previously growing in the RBGE: *B. angulosum* (Rock Garden), *B. fruticosum* (in 3 different places, two on the S side of the Herbarium/Library Building), *B. gibraltarium* (beside the entrance to the Glasshouse Experience), *B. mundii* (Alpine Yard), and *B. salicifolium* (inside the Glasshouse Experience). From my material, 3 other perennial/shrubby species have been added to the collection: *B. ranunculoides* (Acc. No 181), *B. falcatum* s.l. (Acc. No 118), and *B. fruticescens* L. subsp. *spinosum* (Acc. No 155 & 169).

Table 5.1 - Germination data and seedling characters in some species of Bupleurum.

Species were cultivated at ICMB, Edinburgh University (1995) and the Jardim Botânico, Coimbra University (1994). Some species germinated only in Edinburgh (1) or in Coimbra (2). Order of species follows Wolff (1910), synonyms were considered. When first leaves attenuate in a petiole, its length (pt) and that of the remaining limb (lb) are indicated separately. The sign (#) means that just one seedling was produced or that all seedlings died shortly after germination and therefore no voucher specimen was collected.

Cultivated Material		Germination		Hypocotyl		Cotyledons		First Leaves	
TAXON	Total No of Acc. sown	Success (out of total)	Speed (days)	Length (cm)	Colour	Length (cm)	Colour	Length (cm)	Limb Shape
B. rotundifolium	9	9/9	8-15	1-3	brownish	1.5-3	bright green or slightly glaucous	pt 1-3 lb 2-2.5	ovate-lanceolate, shortly-mucronate
B. lancifolium	6	1/6	± 60	1-2	brownish	2-2.5	green slightly glaucous	pt ± 1 lb 2-2.5	ovate-lanceolate, shortly mucronate
B. longifolium (2)	2	1/2	± 30	(#)	(#)	± 2	green slightly glaucous	pt ± 1 lb ± 2.5	ovate-lanceolate, shortly mucronate
B. stellatum	6	0/6	---	---	---	---	---	---	---
B. angulosum	4	0/4	---	---	---	---	---	---	---
B. baldense	4	3/4	10-20	0.3-0.5	green slightly brownish	0.5-0.7	bright green	pt 0.5-1.5 lb 0.5-1	suborbicular to ovate, shortly mucronate
B. praealtum	4	3/4	8-15	2-2.5	reddish/ brownish	1.5-4	bright green	2-7	linear-lanceolate, acuminate
B. gerardii	5	5/5	8-12	± 1	reddish	1-1.5	bright green	1-3.5	linear-lanceolate, acuminate
B. affine	1	1/1	± 15	(#)	(#)	(#)	(#)	(#)	(#)
B. tenuissimum (1)	2	1/2	12-15	0.3-0.5	green slightly brownish	± 1	glaucous	2.5-4	linear-lanceolate, acuminate
B. ranunculoides	17	9/17	20-60	0.3-0.5	reddish	0.6-1.2	green slightly glaucous	pt 0.7-1.3 lb ± 1	linear-lanceolate to obovate-lanceolate, shortly-mucronate
B. longicaule	3	3/3	15-60	0.3-0.5	brownish	0.6-1.8	bright green	pt 1-3 lb 0.6-1	ovate-lanceolate, shortly-mucronate
B. falcatum (1)	22	1/22	15-30	± 0.5	reddish	1-1.5	bright green	pt 1-3 lb1-2	ovate-lanceolate, shortly-mucronate
B. rigidum ssp. ***paniculatum*** (1)	4	2/4	30-90	± 0.1	brownish	3-5	glaucous	3-	linear-lanceolate to ovate lanceolate
B. fruticescens ssp. ***spinosum***	5	5/5	20-60	± 0.5	glaucous	0.5-0.8	glaucous	1-2	obovate-lanceolate, shortly mucronate
B. salicifolium (2)	2	1/2	15-20	± 1	brownish	1-1.7	brownish	1.5-2	lanceolate acuminate
B. canescens *var.* ***handiense*** (2)	1	1/2	20-30	± 1	brownish	1-1.5	green slightly glaucous	pt 0.5-0.8 lb 1-1.5	ovate-lanceolate, shortly mucronate
B. fruticosum	10	7/10	20-90	0.4-0.7	brownish	1-1.5	deep green	pt ± 0.5 lb 0.8-1.5	ovate, shortly mucronate, with narrow scarious margin
B. gibraltarium	2	0/2	---	---	---	---	---	---	---

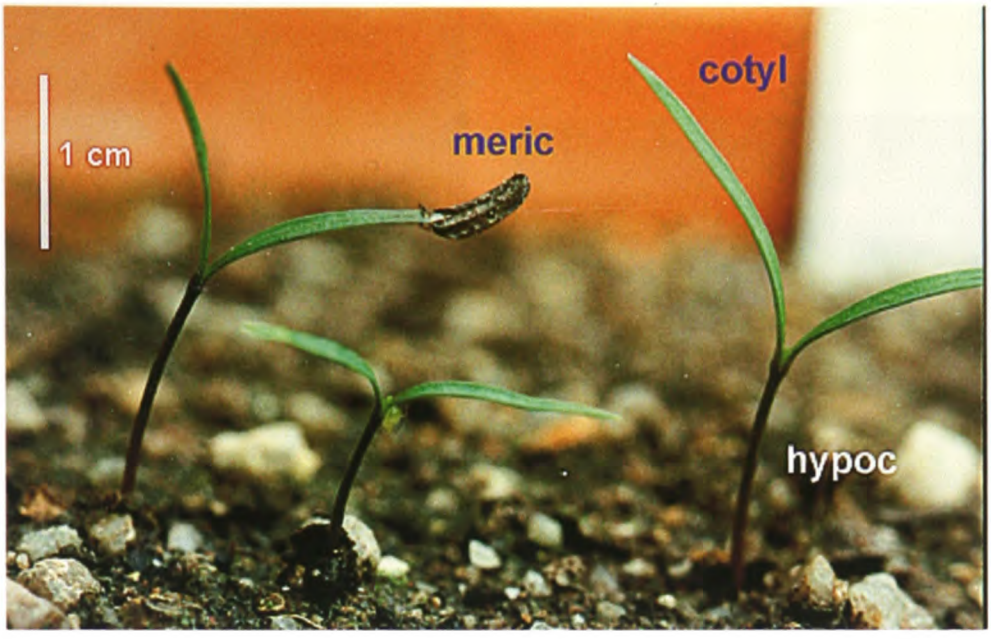


Fig. 5.1 – Seedlings of *Bupleurum canescens* var. *handiense* (Acc. No 28).
Notice the 1-veined linear cotyledons (cotyl) characteristic of the genus and the brownish hypocotyl (hypoc); a mericarp (meric) is still attached to one of the cotyledons.

Fig. 5.2 – Seedlings of *Bupleurum gerardii* (Acc. No 44).
At this stage, first leaves (1st leav) have already been produced. The very narrow linear cotyledons (cotyl) are difficult to see in the image.

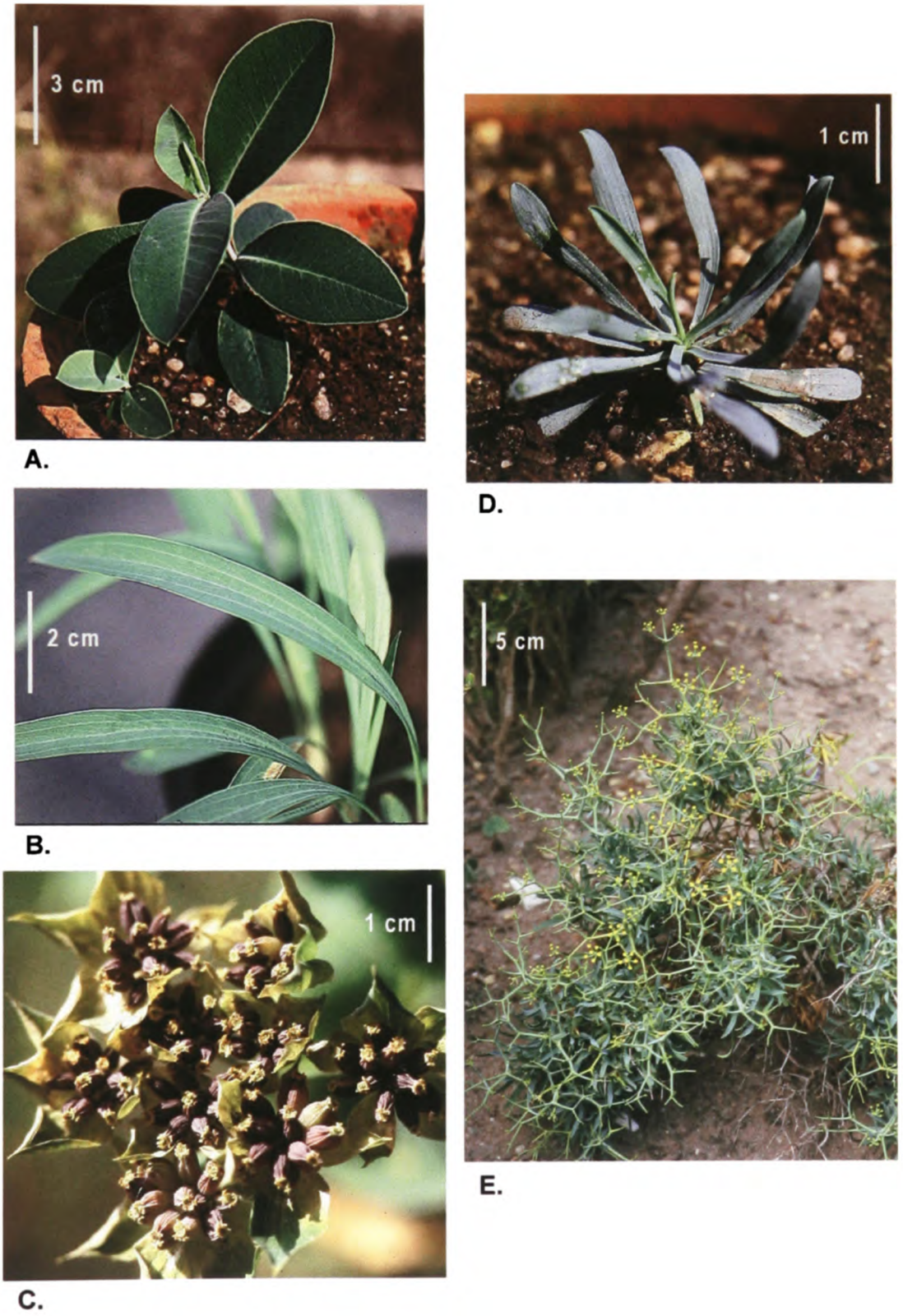


Fig. 5.3 – *Bupleurum* under cultivation. **A. -** Young plants of ***B. fruticosum*** (Acc. No 61), a species with pinnate-reticulate leaves. **B. - *B. falcatum* s.l.** (Acc. No 118); notice parallel venation. **C. -** Fruiting specimen of ***B. rotundifolium*** (Acc. No 13). **D-E. *B. fruticescens* subsp. *spinosum*** (Acc. No 34).; **D.-** seedling; **E.-** mature plant growing at the Botanic Garden of the University of Coimbra.

5.4 Conclusions

1) Fruit collections of *Bupleurum*

My experience of cultivation was limited by the reduced number of taxa of *Bupleurum* available from seed, with most of the critical taxa unaccounted in the collections, in particular N African taxa.

Recent expeditions to NW Africa (conducted by Reading and Sevilla Universities), in particular to Morocco, have enlarged the herbarium collections for the area. Some of these trips took place during June and July to collect late flowering plants (e.g. most of the umbellifers). However, this is still too early to obtain mature fruit collections for most of the species of *Bupleurum*, in particular from those endemic in the area. I have seen part of the herbarium material of *Bupleurum* of these collections, and fruiting specimens are rare if not absent for some of the taxa. The ideal time for collecting fruits should be September and October.

My own collecting trip in the S Iberian Peninsula (August and early September 1997) failed on the same grounds: fruits were not yet ripe. But, later it would have been much more difficult to find the plants, specially the herbs, because, by the time the fruits are fully ripe: a) the characteristic yellow flowers would no longer be visible (petals fall during fruit development), and b) leaves would have degenerated. Also, I would have been unable to collect fresh material for study.

Ideally an initial trip is needed to survey the area, locate populations and tag plants, then followed by later trips for collection of fruits.

2) Germination

Bupleurum fruits are apparently difficult to germinate. However, I am convinced that the main reason for the failure of germination is the poor quality of the fruits, with many not properly developed or may be too old.

Many of the samples I used came from cultivated material in Botanical Gardens. Unfortunately, in many places, growing in greenhouses or in the open garden does not provide the ideal environment for species adapted to very different climates. So, even when flowering actually occurs and fruits seem to mature, they will be of poor quality, often completely lacking endosperm (the embryo is in the middle of this tissue). Umbellifers in general do not have specialized pollinators, and are visited and pollinated by a large array of insects (Bell, 1971). This is likely the

case in *Bupleurum*, but little is known about pollinators in the genus, so the need for particular insects for successful fruit development in botanical gardens cannot be ruled out.

3) Seedling morphological characters

The morphology of the cotyledons in *Bupleurum* gives additional support to the view of the genus as a natural group. Cotyledons are always linear, 1-veined and glabrous in all taxa studied by myself.

First leaves show more diversity, but as many species show similar type of first leaves, identification of most of the taxa is not possible. Maybe the same type of first leaves is an indication of closer relationship, but further research is necessary as the number of taxa so far studied is still small.

6. Investigation of morphology

6.1 Material & methods

6.1.1 Sources of plant material

Most of the morphological study was based on herbarium material, but also, for some species, on observations of cultivated (see chapter 5) or wild plants (fieldwork in the Iberian Peninsula).

For the Iberian taxa, more than 3500 herbarium specimens were studied (c. 3300 obtained on loan) from all the 'basic herbaria' for *Flora iberica* (Castroviejo *et al.*, 1986-cont.). Loans of all Iberian & Balearic material were obtained from BC, BCF, GDA, JACA, MA, MAF, MGC, SALA, SALAF & SEV; and also part of the material in G. The material at COI, another 'basic herbarium', was studied *in situ*. Also, all *Bupleurum* material was consulted during visits to AVE, LISE, LISI & LISU – see Appendix I for names and addresses of herbaria. Codes and information on herbaria follow *Index Herbariorum* (Holmgren *et al.*, 1990).

For the study of NW African taxa, herbarium material (c. 500 specimens) was obtained on loan from BC, BM, K, RNG & SEV (the loan from BM & K was selected during visit to these institutions - 1998). NW African material, in particular, was also studied during visits to MPU and P, in 1996 and 1998 respectively. For the study of Macaronesian (Canary Islands & Madeira) taxa, loans were obtained from BM, K, LISU & LISE (material selected during visit), and also from BC, RNG, TFC & TFMC.

Not only all W Mediterranean and Macaronesian material in the herbarium of the Royal Botanic Garden Edinburgh (E) was revised, but also, although obviously with less detail, the rich collections from the E Mediterranean and Asia, which gave me a better understanding of the diversity and character variation in the genus.

Type material of Linnaean species was studied *in situ* in LINN and BM (herb. Clifford), and also from IDC microfiches of the herbaria of Burser (UPS) and Linnaeaus (S). Type material of *B. atlanticum* Murb. was obtained on loan from P, after being uncovered during visit to this institution. Type material of *B. canescens* Schousb. and *B. lancifolium* Hornem. was also obtained on loan from C.

6.1.2 Methods

The first stage of any taxonomic study is to learn to recognise the different species/taxa based on the morphological characters that have been used in the literature, from the original description (*protologue*) of the species/taxon to the most recent taxonomic treatments – see section 10.2.1 for citation of the main taxonomic references used in the present work.

For the Iberian (or European) species, the number of Floras and other floristic accounts including descriptions and keys to identification is fairly large. Naturally, this made recognition of these taxa much easier, even though after deeper study, some of the characters were considered unreliable.

There is no published floristic account including all the NW African species of *Bupleurum*, not even a complete key to identification of the species (the *Umbelliferae* are yet to be published in the '*Flore de l'Afrique du Nord*', originally edited by R. Maire). Cauwet (1976), in her PhD thesis on the taxonomy of *Bupleurum*, included all the species in the area, but mostly concentrated on her subgenus *Tenoria* (see chapter 2). However, she seemed to accept many of the species with little questioning, even when she was unable to find characters to distinguish them [cf. *B. oligactis* Boiss & *B. atlanticum* Murb. in Cauwet (1976, vol. 3, p. 44-45), and Cauwet & Carbonnier (1977)]. For many of the endemic NW African taxa, the protologue was the only reliable source of information, but in many cases provided no further clarification. As a result, it became imperative to revise the type material of these taxa, which was mainly done during visits to the Montpellier (MPU) and Paris (P) herbaria – both very rich in N African material. The type material of all the other delimited species in this study was also studied.

After the species/taxa have been recognised, it was necessary to assess the diagnostic value of the characters. Ideally, a large number of specimens from all the range of distribution of the species/taxa should be studied. Unfortunately, for some species (often those requiring more study), the number of specimens available was very small (the case for several of the endemic NW African taxa).

It was also important to look for new, potentially useful, characters, not only for identification, but also on the study of taxa relationships. Such new characters, if

available, can be more easily found after the morphology of the species becomes more familiar to us.

6.2 Characters and character states

Diagnostic value of several morphological characters, their reliability and potential interest in the classification of *Bupleurum*, are discussed below.

Habit

Bupleurum includes shrubs, subshrubs, perennial herbs and annuals.

The distinction between a shrub and a subshrub (basically of size/height) is subjective, and is obviously affected by the age of the specimen. However, if fully mature specimens are available, ranges of maximum height can be helpful to distinguish some species in the field, but is of little use in herbarium specimens (height of the plant is only rarely recorded).

It may appear that it is fairly easy to distinguish a shrub (of any size) from a herbaceous plant: a shrub has aerial woody stems, while a herb does not (a perennial herb having only a woody rootstock). But a particular group of *Bupleurum* species [e.g. *B. acutifolium* Boiss, *B. barceloi* Coss ex Willk. and *B. oligactis* Boiss – see chapter 10], which have been often regarded as herbs, are more accurately described as varying from a typical perennial herb to a subshrub. Some herbarium specimens clearly show a short branching woody base, despite most of the stems being herbaceous, and wither after flowering. My observations in the field (*B. acutifolium*) also confirmed this range of variation in habit.

Stem

Characteristics of the stem, such as texture (woody/ herbaceous) and height (or length), are used when we define the habit of a plant. Other morphological characters of the stem have also been used in *Bupleurum*:

a) Basal leaf remains: ***i)*** absent or few; ***ii)*** persistent. This character (maybe more properly regarded as a leaf character) is not of great use to distinguish the species of the area under study, but it appears to be relevant in the diagnostic of some European perennial herbs (see Tutin, 1968).

b) Ridges: prominence and texture (rough/ smooth). These characters were considered critical in the distinction of *B. oligactis* Boiss. and *B. atlanticum* Murb. (see Murbeck's protologue of the latter species; reference in chapter 10). According to Murbeck, the main difference between these species is that *B. atlanticum* has very prominent and 'verrucose' ridges (both characters are related, at least in definition, as rough edges are in this case more prominent than smooth). However, these characters present considerable variation, even within a single specimen (lower stems are generally more 'verrucose'). Rough ridges are found in other taxa (e.g. *B. fruticescens*) where the character is also highly variable. These features are not reliable diagnostic characters as they may be influenced by the environment.

c) Type of ramification (little to much branched). Type of branching is particularly hard to define; some particular branching patterns appear to exist, but growing conditions considerably affect development, resulting in far too much variation for reliable use.

Leaves

a) Texture: ***i)*** herbaceous; ***ii)*** coriaceous. Although useful up to a certain degree, it is subjective, especially because variation in texture is rather continuous among the taxa. Also, in some species leaf texture appears to be affected by growing conditions; leaves of plants growing exposed are often more 'rigid' that those growing under the protection of a wood (possibly the case in *B. salicifolium* R.Br. ex Buch).

b) Leaf size (length & width). In general, quantitative characters have only a limited diagnostic value, as ranges of variation overlap between species. Also, size may be affected by growing conditions.

c) Leaf shape is important in the characterization of taxa, and it can be fairly constant within species (we often need to distinguish shape of basal from that of upper leaves). However, remarkable variation in shape of basal leaves is found between populations of single species (*B. rigidum* L. is the most paradigmatic example – see chapter 10). There are also cases where is basically the upper leaves that show more variation within a species (the case of *B. ranunculoides* L. – C European populations). Therefore, we should be careful when using shape features as diagnostic characters or when establishing relationships.

d) Leaf edge. All *Bupleurum* species show a (very) narrow marginal band, hyaline or ± opaque, which can be: ***i)*** smooth; or ***ii)*** minutely serrulate/ crenulate. There is however a continuous variation between these two states, sometimes even in a single plant. But for some species, it may be used as a last resort character to identify incomplete material (e.g. in the absence of flowering stems, *B. lateriflorum* Coss. may be mistaken for *B. canescens* Schousb. as the leaves are rather similar in shape, but leaf edge is always minutely serrulate in the former, while smooth in the latter).

e) Leaf apex. In most of the species, the leaves taper ± gradually towards the tip (apex acute or acuminate), an obtuse apex is rare. The leaves of several species also show a short mucro, which can either be straight or uncinate. The presence or absence of a mucro is fairly reliable in some species, but the character should be used with care, especially if there is high variation in leaf shape.

e) Veining: There are two main types of venation in *Bupleurum*: ***i)*** *parallel*, and ***ii)*** *pinnate-reticulate*. Distinction between these two main types is clear, but there is considerable variation within what we generally designate as parallel veining, especially because, depending on leaf shape, the veins are sometimes not 'parallel' but curved. There are specific terms for these types of veining (*acrodrome* and *campylodrome* – see e.g. Stearn, 1983), but I will be only using the term parallel for simplification.

Number of veins (only meaningful in parallel veining): Together with details of other leaf characters, such as [maximum] width and shape, number of parallel veins can help identification, but is in general not a reliable character. Number of veins is correlated with width of the leaves: broader leaves simply show more veins (being parallel or not, there is a physiological need to supply the increased leaf tissues). In some taxa the expansion in width, is accompanied by an increase in the number of secondary lateral veins (slender cross-veins). Some taxa do not appear to ever show these lateral cross-veins (there is simply an increase in the number of main veins); while in others the lateral veins are only present in wider leaves. However, variation in width of leaves appears to be affected by environmental conditions; it is not unusual that leaves of exposed plants are narrower than those growing in more shady places (possibly explaining part of the variation in

B. salicifolium). Unfortunately, number of (parallel) veins have been given too much weight in the description/distinction of NW African species.

Prominence of veins: The prominence of the midrib has sometimes been used as a diagnostic character (e.g. between *B. praealtum* L. and *B. gerardii* All.). Some specimens of *B. praealtum* show a thick midrib raised on the abaxial face of the leaves, but in others midrib prominence is basically the same as that in *B. gerardii*. This feature is also variable in other species, and is therefore not reliable. In *B. rigidum*, the main parallel veins are always very prominent (especially in the basal and lower leaves), and many of the secondary cross-veins (when present) are also very thick (unique in the genus). So, in this case vein prominence is a good character for identification.

Intramarginal vein: One species, *B. rigidum*, shows a clear prominent intramarginal vein. The presence of a marginal vein is not exclusive to *B. rigidum*, but, what is special in this case, is that this thick vein runs parallel to the border of the leaf (immediately inside or very near to the narrow marginal band), accompanying the margin to almost the tip, even in different shaped leaves. In other species, a similar vein (generally slender) may run near the margin for a while, but not for all the length of the leaf.

Basal leaves

a) Presence/ absence during flowering. This character is sometimes helpful, but in certain conditions basal leaves may take longer to wither, or the base of the specimen may not be present at all (we may be deceived when recording this).

b) Insertion: ***i)*** sessile; ***ii)*** petiolate. The distinction between sessile and petiolate leaves is rather artificial. In many species it clearly depends on shape of the leaf rather than on any clear morphological distinction between the blade and 'petiole'. For example, in the typical material of *B. rigidum* subsp. *rigidum*, the leaf blade attenuates rapidly towards the base into a more or less long 'petiole', but in *B. rigidum* subsp. *paniculatum* the leaves are generally linear or linear-lanceolate, gradually attenuating to the base (with no 'petiole'); intermediate material between the two subspecies shows all range of variation between these two types of leaf insertion. It is only in the species with pinnately veined leaves that a distinct (parallel-veined) petiole, generally very short, may sometimes be distinguished.

Cauline leaves

Insertion (upper leaves): ***i)*** subamplexicaul; ***ii)*** cordate-amplexicaul; ***iii)*** perfoliate. A rather continuous variation from subamplexicaul to clearly cordate-amplexicaul leaves is found between populations of single species (e.g. *B. ranunculoides*, including C European material), and so these states are not clearly defined. Perfoliate insertion is however a very reliable character-state, which is correlated with other clearly distinct features (such as absence of bracts or lack of *vittae* in the fruits), so it seems to be a good indicator of close relationship.

Inflorescence

a) Ramification of flowering stems: In one species, *B. fruticosum*, there are (generally) only terminal umbels, i.e. the flowering stem is topped by an umbel, and below there are no branches producing secondary 'lateral' umbels [the designation of 'lateral umbel' may be confusing, as all umbels are terminal to a stem, which can sometimes be very short]. However, I have collected a few specimens of *B. fruticosum* that do have lateral umbels (see chapter 10). So, this character is not as reliable to identify the species as has been previously thought (in particular to distinguish *B. fruticosum* from *B. gibraltarium* that always have lateral umbels). In some species there are distinct patterns of ramification of the flowering stems (see e.g. *B. album* Maire, *B. lateriflorum* Coss. ex H.Wolff, and *B. subspinosum* Maire & Weiller – chapter 10). But, for most of the species the pattern is more variable, and generally not reliable for identification, as environmental conditions may considerably affect development. For example, *B. rigidum* can show an extraordinary variation in the amount/pattern of branching and length of flowering stems, even within a single population. This variation appears to be mostly the result of contrasting ecological conditions between different habitats, e.g. specimens growing exposed on a hill side facing strong winds from the sea, contrast remarkably with those growing under the protection of *maquis* or a wood.

b) Rays: Number and length of rays can be helpful to recognise some species, but as they show large range of variation, which generally overlap species, they are generally not discriminatory. In many species, relative length of the rays in each umbel (subequal/ unequal) is variable between and within individuals; so in general

this is not a reliable character. However, in a few cases it is not variable, e.g. in *B. baldense* Turra and *B. odontites* L. as the rays are always clearly unequal.

c) Bracts & bracteoles: The presence/ absence of bracts is a very reliable and an easily recognized feature. Even in the few cases where bracts fall early, we are able to notice the remaining scars. Absence of bracts is exclusive to the '*Perfoliata*' group (see chapter 2 and 10). Despite some degree of variation, number, shape and apex of bracts and bracteoles are fairly reliable characters for identification of species. However, they are not reliable to establish relationships, as species that appear to be unrelated by many other features, sometimes share quite similar bract and bracteoles.

Flowers

a) Number (per umbellule): This is a moderately useful character; some species have very few flowers (1-6) per umbellule, while others can have many (up to 25 or more); but again number ranges generally overlap species boundaries.

b) Pedicel: Length of pedicel has been used as a distinguishing character, in particular in NW African species. However, with a few exceptions (e.g. *B. album*), this character appears to be very unreliable. An additional problem occurs if we are studying specimens in early flowering: the pedicels may appear to be quite short, but they may elongate during flower development and fruiting.

c) Petals: *Bupleurum* flowers (petals) are generally yellow, but in many cases they are tinted with green, purplish or pinkish tones; apparently, there is even a species with 'white' flowers, *B. album* (endemic in Morocco). But of course, colour is not preserved well in dry specimens, and even from fresh material, colour charts would have to be used for accuracy in recording. Also, colour varies during flower development, and appears to differ between populations of the same species (e.g. *B. ranunculoides* – bright yellow or purplish). Other petal characters, such as width, apex ornamentation, and inflexed lobe features (shape, length, maximum width), were particularly useful in distinguishing the annual Turkish species (see Snogerup, 1972). However, the species currently under study in general do not show any clearly distinctive petal characters (with exception, again, of *B. album*, with its unique fimbriate inflexed lobe).

Fruits (see also section 7.1)

a) Shape & size (length and width): There are considerable differences in fruit size and shape between a few species of *Bupleurum*, but the vast majority show very similar fruits.

b) Ornamentation: Most species in *Bupleurum* have smooth fruits, but the few that have papillose or tuberculate fruits are so distinctive that they can be identified by only examining the fruits (e.g. *B. lancifolium* or *B. tenuissimum*).

c) Ridges: They are generally visible, sometimes slightly winged, or very rarely inconspicuous. In a few species, e.g. *B. falcatum* (see chapter 10), ridges can help to identify some material, but in general they do not clearly distinguish the taxa.

Seedlings

For discussion on seedling features see chapter 5 (section 5.3B).

Summary:

With a few exceptions (e.g. veining in *B. rigidum* or the inflorescence in *B. album*), most of the species do not show singular distinguishing features, but rather it is the combination of several particular characters and character-states (generally varying within some range) that discriminate the species from each other – this is not unusual within species of a single genus. But of course, unique characters will not help us to establish relationships; we need reliable and distinct characters that are shared by different species. Unfortunately, there are very few of these characters in *Bupleurum* (e.g. perfoliate leaves and absence of bracts), and so morphology does not provide many clues to define 'natural' groups. Also, several characters (leaf shape, length and number of rays, etc) show considerable variation within single species, and should be used with care when delimiting taxa.

6.3 Numerical analysis using PANKEY and NTSYS programs

6.3.1 Introduction

Numerical taxonomic methods started to develop in the 1960s as the result of increasing criticism on the principles and practices of traditional taxonomy. Unlike other biological disciplines, traditional taxonomy was viewed as having "little advanced from that of a hundred, or even two hundred, years ago", being still fundamentally "intuitive" (Sokal & Sneath, 1963). These mathematical methods aimed to produce classifications that represented more accurately the physical (phenotypic) characters of living organisms, without the much speculation generated by the theories of evolution of the taxa – these methods became also known as **phenetics**.

It is well beyond the aims of this project to properly introduce numerical methods, or to discuss the polemics generated around the phenetic and phylogenetic approaches of analysis (see also section 9.1.2). For detailed explanation and ample discussion on numerical taxonomy see Sneath and Sokal (1973) and Pankhurst (1991).

In the present work, phenetic analysis of character data in *Bupleurum*, was carried out using two computer programs: **PANKEY** (Pankhurst, 1995a), and **NTSYS-pc** - Numerical Taxonomy and Multivariate Analysis SYStem for personal computer (Rohlf, 1992).

PANKEY is a package of programs that allows the analysis of taxonomic data and provides several tools that facilitate the work of taxonomists, such as the automated generation of descriptions or keys to identification after a matrix of character data in standard format (DELTA - DEscription Language for TAxonomy) has been provided. For this work, PANKEY was used to:

1) Create a character data file for the taxa in DELTA format (using the Microsoft editor and DELTA editor programs).

2) Generate *similarity coefficients* - a similarity value is calculated between every two taxa and between all. This matrix of similarity coefficients can then be used for clustering in other programs, such as NTSYS.

NTSYS-pc is a package of programs which includes clustering and ordination methods of phenetic analysis. Clustering methods produce *dendrograms* (= *phenograms*), which graphically represent the level of similarity expressed by the data. Ordination methods are alternative geometrical methods that avoid the assumption that clusters are present (see Sneath & Sokal, 1973, chapter 5; and Pankhurst, 1991, p. 56-64).

6.3.2 Selected characters and character states

Characters were selected fundamentally because they showed variation on the species/taxa under study, and were in many cases essential for identification. These characters were recorded during my study of *Bupleurum*, so there was no predetermined number of specimens to be measured. This number largely depended on the herbarium material available for each taxon: for example, only 8 specimens were studied for *B. subspinosum* Maire & Weiller, while hundreds were available for *B. rigidum* L.

The following text is the data matrix in DELTA format used for analysis. For detailed explanation on DELTA format see Pankhurst (1991, p. 110-116) and the PANKEY manual available with the program.

Delta files include two kind of information: *directives* and *data*. Directives give the parameters of the file, and divide the data into sections, starting with an asterisk (*). *Character types* are: UM = 'Unordered Multistate' (not arranged in natural order) – set as default; OM = 'Ordered Multistate' (in 'natural' order); IN = Integer Numeric (integers); RN = Real Numbers. *Key states* can be used to split quantitative characters in ranges, i.e. as if they were qualitative states. Some characters are logically dependent, e.g. if bracts are absent there is no sense in asking for bract shape; characters of this type are indicated in the directive *dependent characters*. Other symbols or abbreviations used are:

- beginning of new character

/ - end of character or character state; or, when scoring the data, two or more states are present in a single taxon.

< > - comment

U - unknown character

V - variable character

Data matrix in DELTA format

*HEADING BUPLEURUM/
*NUMBER OF CHARACTERS 42
*MAXIMUM NUMBER OF STATES 8
*MAXIMUM NUMBER OF ITEMS 31
*CHARACTER TYPES 2,RN 3,OM 5,RN 6,RN 7,OM 21,IN 22,RN 27,RN 28,RN 32,
IN 36,RN 37,RN 39,OM
*NUMBER OF STATES 3,4 7,6 8,3 9,4 11,3 12,3 13,4 15,3 16,6 17,3 19,3 23,3
29,6 30,5 33,3 34,3 35,3 38,3 39,4 41,8 42,4
*CHARACTER WEIGHTS <all equally weighed>
*KEY STATES <not used>

*CHARACTER DESCRIPTIONS

#1. Habit/
1. annual/
2. perennial/

#2. Height <or maximum length if decumbent>/ cm/

#3. Stems <texture>/
1. <totally> herbaceous/
2. herbaceous but with woody rootstock/
3. with short woody branches at base, the rest herbaceous>/
4. becoming woody/

#4. Leaves <texture>/
1. herbaceous <soft to the touch>/
2. coriaceous <more or less rigid>/

#5. Leaves <length>/ cm/

#6. Leaves <width> cm/

#7. Leaves <shape>/
1. linear to linear-lanceolate/
2. lanceolate/
3. oblong-lanceolate/
4. obovate/
5. ovate/
6. suborbicular/

#8. Leaves <apex shape>/
1. acuminate/
2. acute/
3. obtuse/

#9. Leaves <apex direction>/
1. with straight apex/
2. with straight short mucro/
3. with uncinate apex/
4. with uncinate short mucro/

#10. Basal leaves <presence at flowering time>/
1. present/
2. withering before flowering/

#11. Basal leaves <base>/
1. not attenuate or sligthly attenuate to the base/
2. gradually attenuate to the base/
3. abruptly attenuate into more or less long petiole/

#12. Cauline leaves <spacing along the stem>/
1. sparse/
2. crowded/
3. in clusters/

#13. Upper leaves <insertion>/
1. subamplexicaul/
2. cordate-amplexicaul/
3. perfoliate/
4. very shortly petiolate/

#14. Leaves <veining>/
1. pinnate-reticulate/
2. parallel/

#15. Secondary veins <presence>/
1. absent/
2. few/
3. numerous and reticulate/

#16. Leaf veins <prominence - upper leaves excluded>/
1. visible but not raised/
2. all slightly raised on abaxial surface/
3. all slightly raised on both surfaces/
4. <basically> all very prominent/
5. only midrib raised on abaxial surface <lateral reticulate veins>/
6. only midrib raised on abaxial surface <parallel veins>/

#17. Flowering stems <branching>/
1. unbranched/
2. little branched/
3. much branched/

#18. Umbels/
1. terminal <exceptionally lateral present>/
2. terminal and lateral/

#19. Lateral umbels/
1. long pedunculate/
2. short pedunculate/
3. sessile or subsessile <axillary>/

#20. Lateral umbels <no. rays>/
1. <always> 1-rayed/
2. with one to more rays/

#21. Rays <no.>/

#22. Rays <length>/ cm/

#23. Rays <texture>/
1. slender/
2. thick/
3. stiff and spinescent/

#24. Bracts <presence>/
1. present/
2. absent/

#25. <Bracts-Rays relative length>/
1. bracts shorter than rays/
2. bracts of similar length or longer than longest ray/

#26. Bracts <posture>/
1. erect to patent/
2. reflexed/

#27. Bracteoles <length>/ mm/

#28. Bracteoles <width>/ mm/

#29. Bracteoles <shape>/
1. linear/
2. linear-lanceolate/
3. lanceolate/
4. oblong/
5. ovate/
6. suborbicular/

#30. Bracteoles <apex>/
1. obtuse/
2. acute/
3. acuminate/
4. mucronate/
5. aristate to apiculate/

#31. Bracteoles <texture or substance>/
1. membranaceous or translucent/
2. opaque/

#32. Flower <no. per umbellula>/

#33. Petal <colour>/
1. whitish/
2. yellow to yellowish-green/
3. purplish-green/

#34. <Petal> mid vein <colour>/
1. colour the same as rest of the petal/
2. light brownish/
3. blackish/

#35. <Petal> inflexed apex <margin>/
1. entire or subentire/
2. two-lobed/
3. fimbriate/

#36. Pedicel <length>/ mm/

#37. Mericarp <length> mm/

#38. Mericarp <surface>/
1. smooth/
2. tuberculate/
3. papillose/

#39. Ridges <prominence>/
1. inconspicuous/
2. filiform/
3. prominent/
4. narrowly winged/

#40. Ridges <ornamentation>/
1. smooth/
2. crenulate/

#41. Chromosome number <2n>/
1. 14/
2. 16/
3. 21/
4. 24/
5. 28/
6. 30-31/
7. 32/
8. 42/

#42. Geographical distribution/
1. Iberian Peninsula/
2. Balearic Islands/
3. NW Africa/
4. Macaronesia/

*DEPENDENT CHARACTERS 18,1:19-20 24,2:25-26 39,1:40

*ITEM DESCRIPTIONS

#1.B.acutifolium/
1,2 2,30-100 3,2/3 4,1/2 5,1-16 6,0.1-0.6 7,1 8,1 9,1 10,2 11,1 12,1/2 13,1 14,2 15,1 16,3 17,2 18,2 19,1/2 20,2 21,3-14 22,1-3.2 23,1 24,1 25,1 26,1/2 27,3-5 28,0.2-1 29,1 30,3 31,2 32,5-12 33,2 34,1/2 35,1 36,2-10 37,3-6 38,1 39,2 40,1 41,7 42,1

#2.B.album/
1,2 2,5-40 3,2/3 4,2 5,0.3-2 6,0.15-0.3 7,1/2/3 8,2 9,4 10,1/2 11,1 12,1/2 13,1 14,2 15,1 16,1 17,1 18,2 19,3 20,2 21,1-4 22,0.1-1.9 23,1/2 24,1 25,2 26,1 27,1.5-2 28,0.4-0.5 29,1/2/3 30,1/2 31,2 33,1 34,1/2 35,3 36,0.1-0.2 37,3-4 38,1 39,4 40,1/2 41,7 42,3

#3.B.angulosum/
1,2 2,15-70 3,2 4,1 5,1.5-40 6,0.3-3 7,1/2/3 8,2/3 9,1 10,1 11,2/3 12,1 13,2 14,1 15,3 16,5 17,2 18,2 19,1 20,2 21,3-8 22,1-7 23,2 24,1 25,1 26,1 27,5-20 28,4-15 29,4/6 30,1 31,2 32,10-50 33,2/3 34,1 35,1 36,3-6 37,4-7 38,1 39,4 40,1 41,1 42,1

#4.B.balansae/
1,2 2,15-70 3,4 4,2 5,0.8-8 6,0.1-0.5 7,1 8,1 9,1 10,2 11,1 12,1/2 13,1 14,2 15,1 16,3 17,2/3 18,2 19,1 20,2 21,3-10 22,0.4-5 23,1 24,1 25,1 26,1 27,1.5-4 28,0.3-0.8 29,1/2 30,2 31,2 32,3-12 33,2 34,2 35,1 36,0.3-1 37,3.5-5 38,1 39,2 40,1 41,7 42,3

#5.B.baldense/
1,1 2,2-30 3,1 4,1 5,1-8 6,0.2-1 7,1/2/3 8,1/2 9,1 10,2 11,2 12,1 13,1 14,2 15,1/2 16,1 17,2 18,2 19,1 20,2 21,2-5 22,0.1-2.5 23,1 24,1 25,2 26,1 27,5-14 28,1.5-5 29,3/5 30,5 31,2 32,3-9 33,2 34,1/2 35,1 36,1-5 37,2-2.5 38,1 39,2 40,1 41,2 42,1

#6.B.barceloi/
1,2 2,20-100 3,2/3 4,1/2 5,1-18 6,0.1-0.5 7,1 8,1 9,1 10,2 11,1 12,1/2 13,1 14,2 15,1 16,3 17,2 18,2 19,1/2 20,2 21,3-12 22,0.4-2.6 23,1 24,1 25,1 26,1/2 27,1-3 28,0.5-1 29,1 30,3 31,2 32,3-10 33,2 34,3 35,1 36,3-6 37,4-6 38,1 39,3/4 40,1 41,4 42,2

#7.B.benoistii/
1,2 2,2-15 3,2 4,1 5,1-3.5 6,0.2-0.5 7,2/3 8,2/3 9,1 10,1 11,2 12,1 13,1 14,2 15,1 16,1 17,2 18,2 19,1/2 20,2 21,3-5 22,0.1-2.4 23,1 24,1 25,1/2 26,1/2 27,1-2 28,0.3-0.5 29,1-3 30,2 31,2 32,3-12 33,2 34,2 35,2 36,0.5-1.5 37,4-7 38,1 39,3/4 40,1 41,U 42,3

#8.B.canescens/
1,2 2,100-200 3,4 4,2 5,1-8 6,0.3-3 7,3/4 8,3 9,4 10,2 11,2 12,1 13,1 14,2 15,1 16,2 17,2 18,2 19,1 20,2 21,1-13 22,0.3-4.2 23,1/2 24,1 25,1 26,1 27,2-3 28,0.7-1.5 29,3/5 30,2 31,2 32,5-20 33,2 34,2 35,1 36,1-3.5 37,4.5-6 38,1 39,3 40,1 41,7 42,3/4

#9.B.dumosum/
1,2 2,60-100 3,4 4,1 5,0.5-3.5 6,0.05-0.4 7,1/2/3 8,2/3 9,4 10,2 11,1/2 12,3 13,1 14,2 15,1 16,1 17,3 18,2 19,1 20,2 21,2-6 22,0.6-2 23,1 24,1 25,1 26,1 27,2-3 28,0.3-0.5 29,1 30,2/3 31,2 32,2-8 33,2 34,2 35,1/2 36,0.5-1.5 37,3-4.5 38,1 39,2 40,1 41,7 42,3

#10.B.falcatum/
1,2 2,20-80 3,2 4,1 5,2-18 6,0.3-2.5 7,1/2/3 8,2/3 9,1 10,1 11,3 12,1 13,1 14,2 15,2 16,1/2 17,2/3 18,2 19,1 20,2 21,3-15 22,0.3-5 23,1 24,1 25,1 26,1 27,2-8 28,0.8-1.5 29,1-3 30,3 31,2 32,3-25 33,2 34,1/2 35,1/2 36,1-3 37,2-4 38,1 39,4 40,1 41,2 42,1

#11.B.foliosum/
1,2 2,20-75 3,4 4,2 5,1-10 6,0.2-1.2 7,1/2/3 8,1/2 9,1 10,2 11,1/2 12,1/2 13,1 14,1 15,3 16,5 17,2 18,2 19,1 20,2 21,1-4 22,0.6-4 23,1 24,1 25,1 26,1 27,4-6 28,1-3 29,3/5 30,1/4 31,2 32,3-15 33,2 34,1/2 35,1 36,1.5-5 37,4-6 38,1 39,4 40,1 41,1 42,1/3

#12.Bfrue <B.fruticescens ssp. fruticescens>/
1,2 2,10-120 3,4 4,1/2 5,1-14 6,0.05-1 7,1/2 8,1/2 9,3 10,2 11,1 12,1/2 13,1 14,2 15,1 16,1/2 17,3 18,2 19,1 20,2 21,1-9 22,0.5-4 23,1 24,1 25,1 26,1/2 27,1-5 28,0.2-0.5 29,1 30,3 31,2 32,1-8 33,2 34,1/2 35,1/2 36,1-10 37,3-6 38,1 39,2 40,1 41,7 42,1

#13.B.spin <B.fruticescens ssp. spinosum>/
1,2 2,10-120 3,4 4,2 5,0.5-6 6,0.05-1 7,1/2/3 8,1/2 9,3 10,2 11,1 12,1/2 13,1 14,2 15,1 16,1/2 17,3 18,2 19,1 20,2 21,1-6 22,0.5-4 23,3 24,1 25,1 26,1/2 27,1-5 28,0.2-0.5 29,1 30,3 31,2 32,1-8 33,2 34,1/2 35,1/2 36,1-10 37,3-6 38,1 39,2 40,1 41,7 42,1

#14.Bfruo <B.fruticosum>/
1,2 2,60-300 3,4 4,2 5,1-13 6,0.4-4.5 7,3/4 8,2/3 9,2 10,2 11,2 12,1 13,4 14,1 15,3 16,5 17,2 18,1 21,3-25 22,1-6 23,2 24,1 25,1 26,1/2 27,2.5-8 28,1-3 29,2-4 30,4 31,2 32,5-18 33,2 34,1/2 35,1 36,3-12 37,5-8 38,1 39,4 40,1 41,1 42,1/3

#15.B.gerardii/
1,1 2,20-70 3,1 4,1 5,1-8 6,0.2-0.4 7,1 8,1 9,1 10,2 11,1 12,1 13,1 14,2 15,1 16,1/6 17,2 18,2 19,1 20,2 21,1-10 22,0.2-6 23,1 24,1 25,1 26,1 27,4-9 28,0.5-1 29,1 30,3 31,2 32,1-8 33,2 34,1/2 35,1 36,0.5-3 37,2-3.5 38,1 39,2 40,1 41,2 42,1

#16.B.gibraltarium/
1,2 2,60-200 3,4 5,3-24 6,0.3-3 7,2/3 8,2 9,4 10,2 11,2 12,1/2 13,1/4 14,1
15,3 16,5 17,2/3 18,2 19,1/2 20,2 21,3-57 22,1-13 23,2 24,1 25,1 26,2 27,2-9
28,2-5 29,3/5 30,2 31,2 32,3-25 33,2 34,1/2 35,1 36,2-11 37,4-11 38,1 39,4
40,1 41,1 42,1/3

#17.B.lancifolium/
1,1 2,6-75 3,1 4,1 5,1.5-15 6,1-6 7,2/3/4/5 8,2/3 9,2 10,2 11,2 12,1 13,3
14,2 15,2 16,1/6 17,2 18,2 19,1 20,2 21,2-4 22,0.5-3 23,1 24,2 27,2-16
28,3-15 29,3/5 30,1/2/4 31,2 32,6-25 33,2 34,1 35,1 36,1-4 37,3-5 38,2 39,2
40,1 41,2 42,1/2/3/4

#18.B.lateriflorum/
1,2 2,50-80 3,4 4,1/2 5,0.5-5.8 6,0.1-1.7 7,3/4 8,2/3 9,4 10,2 11,2 12,1 13,1
14,2 15,1 16,1/2 17,1 18,2 19,2 20,2 21,3-11 22,0.3-1.8 23,1 24,1 25,1 26,1
27,1-2 28,0.3-0.5 29,3/5 30,2 31,2 32,3-12 33,2 34,1 35,1/2 36,1-2.5 37,4-5
38,1 39,4 40,1 41,5 42,3

#19.B.montanum/
1,2 2,50-150 3,3 4,1 5,1.3-18.5 6,0.1-1.3 7,1/2 8,2/3 9,1/2 10,2 11,1 12,1/2
13,1 14,2 15,1/2 16,1/2 17,2 18,2 19,1 20,2 21,3-15 22,0.5-4.2 23,1 24,1 25,1
26,1 27,1.5-4 28,0.3-0.5 29,1 30,3 31,2 32,1-20 33,2 34,2 35,1 36,1-7 37,4-7
38,1 39,4 40,1 41,5/6/7 42,3

#20.B.odontites/
1,1 2,5-50 3,1 4,1 5,2-12 6,0.15-1 7,1/2 8,1/2 9,1 10,2 11,1 12,1 13,1 14,2
15,1 16,1 17,2/3 18,2 19,1 20,2 21,3-7 22,0.3-3.5 23,1 24,1 25,2 26,1 27,4-20
28,1-4 29,3/5 30,2/3/5 31,1 32,7-13 33,2 34,1/2 35,2 36,1-8 37,1.3-2.2 38,1
39,2 40,1 41,2 42,3

#21.B.oligactis/
1,2 2,30-80 3,3 4,2 5,1.5-10.5 6,0.12-0.45 7,1 8,1 9,1 10,2 11,1 12,1/2 13,1
14,2 15,1 16,1/2 17,2/3 18,2 19,1 20,2 21,1-6 22,1-6 23,1 24,1 25,1 26,1
27,1-2 28,0.15-0.2 29,1 30,3 31,2 32,4-8 33,2 34,2 35,1/2 36,2-8.5 37,4-5
38,1 39,2 40,1 41,5/6/7 42,3

#22.B.plantagineum/
1,2 2,60-160 3,4 4,2 5,1-16 6,0.2-4.2 7,2/3 8,1/2 9,2 10,2 11,2 12,1 13,1
14,2 15,1 16,1/2 17,2 18,2 19,1 20,2 21,1-20 22,0.4-4.5 23,1 24,1 25,1 26,1
27,3-6 28,0.4-0.7 29,2/3 30,3 31,2 32,5-11 33,2 34,2 35,2 36,3-6 37,4-7.5
38,1 39,4 40,1 41,5 42,3

#23.B.praealtum/
1,1 2,30-150 3,1 4,1 5,2-20 6,0.2-0.8 7,1 8,1 9,1 10,2 11,1 12,1 13,1 14,2
15,1 16,5 17,2/3 18,2 19,1/2 20,2 21,1-7 22,0.6-3.3 23,1 24,1 25,1 26,1
27,1.5-5 28,0.3-1 29,1 30,3 31,2 32,1-6 33,2 34,1 35,1 36,1-3.5 37,4-7 38,1
39,2 40,1 41,2 42,1

#24.B.ranunculoides/
1,2 2,3-45 3,2 4,1 5,0.5-18 6,0.1-1.5 7,1/2/3 8,1/2 9,1 10,1 11,1/2 12,1
13,1/2 14,2 15,1 16,1 17,2 18,2 19,1 20,2 21,1-10 22,0.25-4 23,1 24,1 25,1
26,1 27,2-8 28,1-3 29,3/5 30,1/2 31,2 32,4-25 33,2/3 34,1/2 35,1 36,0.5-3
37,2-4 38,1 39,2 40,1 41,1/3/5/8

#25.B.rigidum <B.rigidum ssp. rigidum>/
1,2 2,12-150 3,2 4,2 5,1-45 6,0.1-6.5 7,1/3/4 8,1/2/3 9,1 10,1 11,3 12,1 13,1
14,2 15,2 16,4 17,2/3 18,2 19,1 20,2 21,1-6 22,0.3-9 23,1 24,1 25,1 26,1
27,1-1.5 28,0.2-0.4 29,1 30,3 31,2 32,1-12 33,2 34,1/2 35,1 36,1-11 37,3-7
38,1 39,2 40,1 41,2 42,1/3

#26.Bripa <B.rigidum ssp. paniculatum>/
1,2 2,12-150 3,2 4,2 5,1-45 6,0.1-1.2 7,1/2 8,1/2 9,1 10,1 11,1/2 12,1 13,1
14,2 15,1/2 16,4 17,2/3 18,2 19,1 20,2 21,1-6 22,0.3-9 23,1 24,1 25,1 26,1
27,1-1.5 28,0.2-0.4 29,1 30,3 31,2 32,1-12 33,2 34,1/2 35,1 36,1-11 37,3-7
38,1 39,2 40,1 41,1/2 42,1/3

#27.B.rotundifolium/
1,1 2,10-85 3,1 4,1 5,1-10 6,1-5 7,3/4/5/6 8,3 9,2 10,2 11,2 12,1 13,3 14,2
15,2 16,1/6 17,2 18,2 19,1 20,2 21,2-11 22,0.3-2 23,1 24,2 27,5-15 28,2.5-14
29,3/5 30,1/2/4 31,2 32,5-15 33,2 34,1 35,1 36,1.5-2 37,2.5-4 38,1 39,2 40,1
41,2 42,1

#28.B.salicifolium/
1,2 2,100-200 3,4 4,1/2 5,1.2-15 6,0.3-2.2 7,1/2 8,1 9,3 10,2 11,2 12,1 13,1
14,2 15,1/2 16,1 17,2/3 18,2 19,1 20,2 21,1-14 22,0.6-4.7 23,1/2 24,1 25,1
26,1/2 27,1-2.5 28,0.3-1.2 29,2/3 30,2 31,2 32,3-12 33,2 34,2 35,1 36,3-6
37,5-7 38,1 39,3/4 40,1 41,7 42,4

#29.B.semicompositum/
1,1 2,2-35 3,1 4,1 5,1-6 6,0.1-0.8 7,1 8,1 9,1 10,1/2 11,2 12,1 13,1 14,2
15,1/2 16,1 17,3 18,2 19,1 20,2 21,2-12 22,0.1-3 23,1 24,1 25,1/2 26,1 27,2-8
28,0.5-1.5 29,1/2 30,3 31,2 32,2-9 33,2/3 34,1/2 35,1 36,0.5-6 37,1-2 38,3
39,1 41,2 42,1/2/3/4

#30.B.subspinosum/
1,2 2,20-40 3,4 4,1 5,0.8-5.5 6,0.2-0.35 7,2/3 8,2 9,1 10,2 11,2 12,1 13,1
14,2 15,1 16,1 17,3 18,2 19,3 20,1 21,1-3 22,0.2-1.5 23,1/2 24,1 25,1 26,1
27,0.3-2 28,0.4-0.6 29,3/5 30,1/2 31,2 32,1-4 33,2 34,1/2 35,2 36,0.1-1.5
37,3-5 38,1 39,2 40,1 41,7 42,3

#31.B.tenuissimum/
1,1 2,4-70 3,1 4,1 5,0.5-8 6,0.05-0.6 7,1 8,1/2 9,1 10,2 11,1 12,1 13,1 14,2
15,2 16,1 17,2 18,2 19,2/3 20,2 21,1-6 22,0.1-2 23,1 24,1 25,1/2 26,1 27,1-6
28,0.3-1 29,1 30,3 31,2 32,1-6 33,2/3 34,1/2 35,1 36,0.5-2 37,1.2-2.5 38,3
39,2 40,2 41,2 42,1/2/3

*END

6.3.3 Results

A phenogram obtained from clustering analysis using the SAHN program (UPGMA method) of the NTSYS package (Rohlf, 1992) is presented in Fig. 6.1 – [UPGMA = *Unweighted Pair Group Method with Arithmetic Means*]. The SAHN (*Sequential Agglomerative Hierarchic Non-overlapping*) clustering methods have been the most frequently used strategy for finding clusters based on matrices of similarity coefficients.

Ordination analysis of the same matrix of similarity coefficients using the NTSYS programs, produced a three-dimensional projection (Fig. 6.2) onto the first three principal components (X, Y and Z axes), with a *minimum spanning tree* superimposed. A minimum spanning tree is a graph that connects each taxon with the next most similar one; the next taxon to be joined would be the closest to any of those already in the graph, but in a way that no two points are connected again, i.e. there can be no loops (Pankhurst, 1991). As Fig. 9.2 is projected in a plan, the three dimensional relation between the taxa cannot be properly seen.

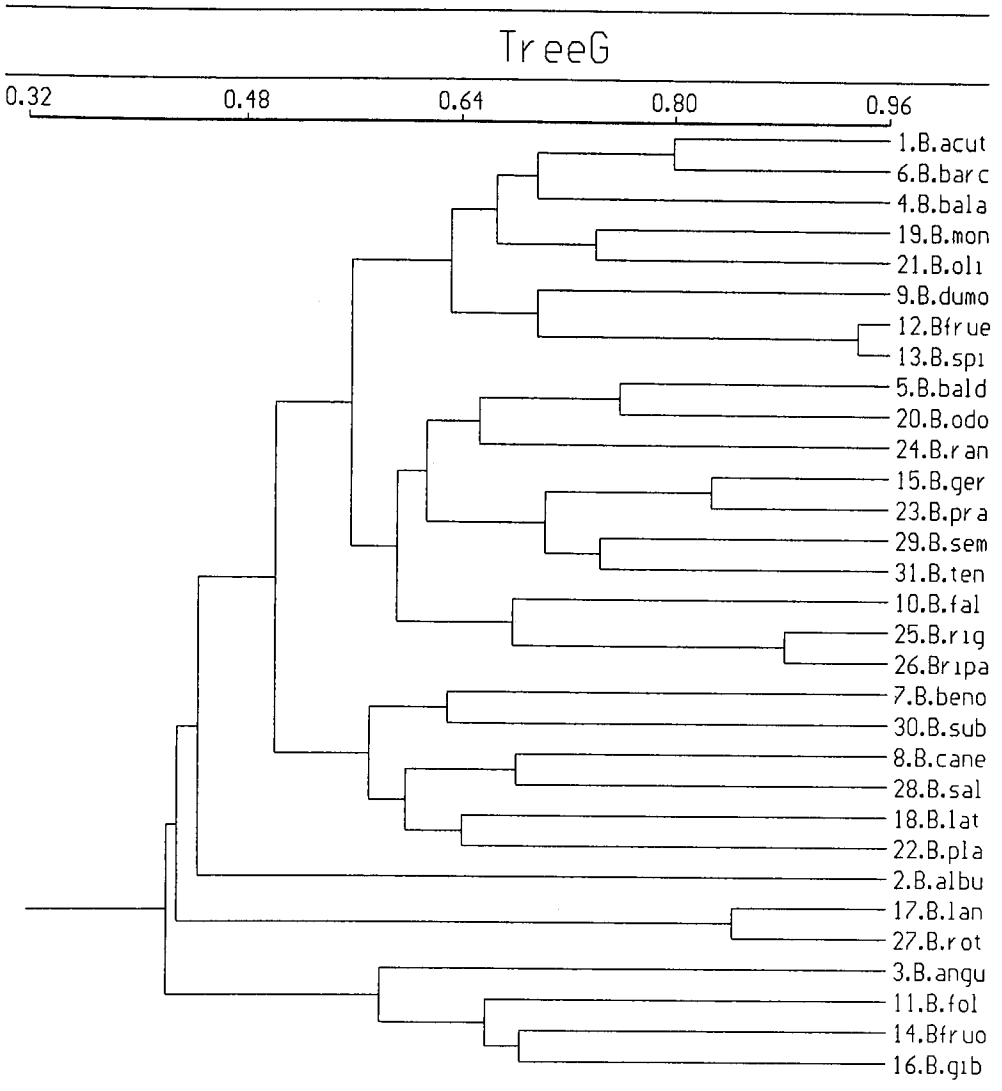


Fig. 6.1 - Phenogram of 31 taxa of *Bupleurum* produced by clustering analysis. A SAHN method (UPGMA) of the NTSYS-pc programs (Rohlf, 1992) was used after a matrix of similarities coefficients was obtained by the PANKEY program (Pankhurst, 1995a). For complete name of taxa, see the matrix of characters in DELTA format ("item descriptions") in section 6.3.2.

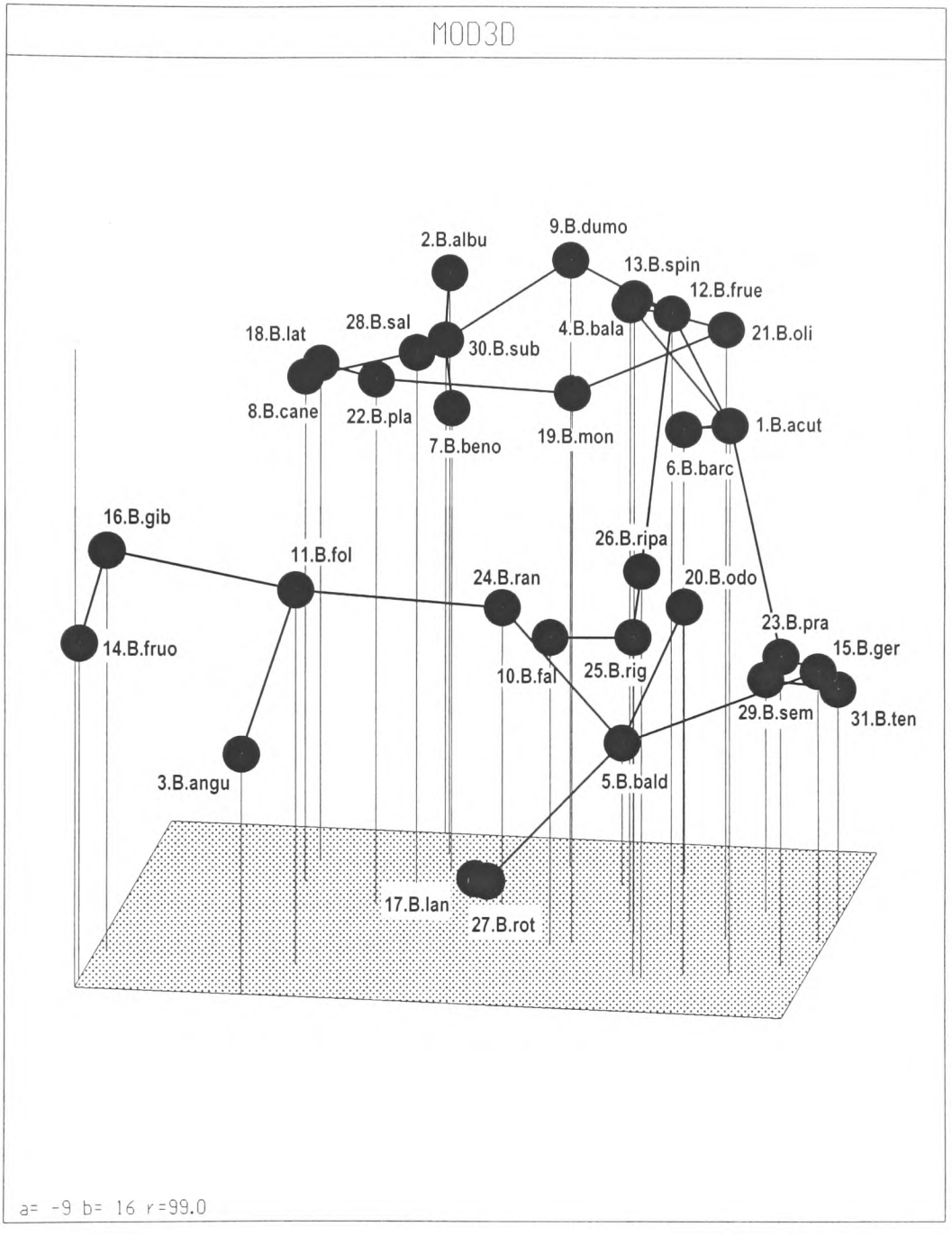


Fig. 6.2 - Three-dimensional projection of 31 taxa of *Bupleurum* produced by ordination analysis. Projection based on the first three principal components from multivariate analysis, with a minimum spanning tree superimposed to the taxa, obtained using the NTSYS-pc programs (Rohlf, 1992). This graph connects each taxon to the next most similar ones; there is some distortion of the distances between the taxa, and so in this view some taxa may appear more close even when there are no lines connecting them. For complete name of taxa, see the matrix of characters in DELTA format ("item descriptions") in section 6.3.2.

6.3.4 Discussion

Although all the taxa that are clearly morphologically similar form groups in the phenogram of Fig. 6.1, I am not satisfied with some of the associations (clusters) that resulted from the analysis. *Bupleurum benoistii* and *B. subspinosum* formed a group, but there are no clear morphological similarities between these species; *B. subspinosum*, as the name suggests, is clearly more similar to '*B. spinosum*' (*B. fruticescens* subsp. *spinosum*). So, in this case, I did not provide enough characters to express the morphological resemblance of these taxa. *Bupleurum baldense* and *B. odontites* have morphological affinities (they have been generally classified in the same taxonomic group), but there is no obvious reason why these taxa should be more closely associated to *B. ranunculoides* than to other herbaceous species. One character that certainly contributed to the unusual association of *B. falcatum* and *B. rigidum* subsp. *rigidum* is that both taxa have petiolate basal leaves. However, as discussed in section 6.2, this is simply not a reliable character as it varies within species. Unfortunately, many of the other characters appear to be unreliable to establish relationships.

One of the reasons for the peculiar associations established between the taxa in the present analysis, may be that I have not provided enough or the 'right' characters. Another explanation is that clustering methods produce 'clusters' whatever the structure of the data, even if the character distributions in the taxa are at random (Sneath & Sokal, 1973). To avoid the assumption that clusters exist, ordination methods are used – see the results of ordination analysis in Fig. 6.2. Some of the lines that connect the closer taxa are not clearly seen in this projection, and the distances between some taxa are distorted. But the program allow us to rotate the graph, horizontally and vertically, to better visualise the lines connecting the closer taxa. This method does not clearly distinguish clusters, but it is possible to notice some spatial association of the taxa. A major group including all the NW African endemics and Macaronesian taxa appears to be visible in the upper part of the graph (this group corresponds to the 'NW African origin' group – see section 9.4); this group was not delimited by clustering analysis. All the other groups in the three-dimensional graph (Fig. 6.2) correspond to those defined in the phenogram of Fig. 6.1.

To represent numerically the existing morphological similarity, we need to provide a very large number of characters. But, as it is simply not possible to measure or record every single morphological detail, it is necessary to select characters. Also, we need to define the character-states, that in many cases represent continuous variation rather than discrete states, i.e. there are no real 'gaps'. This happens with quantitative characters such as length, but also with other characters that might be regarded as qualitative data, e.g. shape of leaves. So, there is some subjectivity when recording data for analysis.

There has been much discussion about the need of having stable classifications, and that this may be achieved if we base our classifications on the characters of the present living organisms rather than on theories of how they may have evolved. However, biologists still expect the classification to reflect what is known about the relationships of organisms. So, in the end, a classification can only be stable if is supported by phylogeny, mere superficial similarity will not do.

A great degree of similarity is often a good indicator of close relationship, but this is not always the case. We know that similarity may have independently arisen in unrelated groups (*convergence*) or in different lineages of more closely related taxa (*parallelism*). It has been argued that providing large numbers of characters will distinguish 'apparent' from 'real' similarity. This may be true for taxa that are more distantly related, where adding more morphological characters may make it clear that the similar ones are the result of convergence. But when we are studying taxa that are closely related, such as the species of a single genus, cases of parallel evolution will be more difficult to detect, as there are obviously a lot of other common characters.

In summary, morphological characters can be misleading about relationships, and analysing the data numerically will not constitute a significant improvement over 'intuitive' reasoning. We need other sources of data to verify relationships suggested by morphology.

In the case of this study, molecular analysis (sequencing of ITS gene region – see chapter 9) uncovered some relationships that were not reflected by morphological similarity (see section 9.4). So, there are significant differences between the present numerical analyses of morphological and molecular data (cf. Fig. 6.1 and Figs 9.4-9.8). These differences have nothing to do with the fact that a phenetic approach was used for morphological data and a phylogenetic (cladistic) approach for the

molecular data. In fact, the molecular data was also analysed using the neighbour joining method (see Fig. 9.7), that as it uses distances (coefficients of similarity or dissimilarity) is sometimes regarded as a 'phenetic' method (see section 9.1.2), and the results were very similar to those of cladistic analysis.

Numerical analysis of phenotypic (morphological) data has no doubt certain advantages, especially because it can make more clear how the grouping of taxa is affected by particular characters. Also, when the number of taxa is very large, numerical analysis can help our taxonomic decisions. However, if we believe that classifications should represent relationships we also need genetic data to verify if there is true homology between similar morphological characters.

7. Investigation of anatomy

7.1 Introduction

Fruit characters have been, and are, extremely important in the taxonomy of the *Umbelliferae*. Some studies have shown some relevant variation in fruit anatomy between species and taxa of *Bupleurum* (e.g. Arenas & García, 1993), so a detailed study of fruit anatomy is appropriate.

The fruit in *Bupleurum*

As for all typical umbellifers of subfamily *Apioideae*, *Bupleurum* has a dry fruit formed by two *mericarps* developed from an inferior bicarpellate ovary. The mericarps are joined at the *commissural face* (or *commissure*) by a central stalk, the *carpophore*, which splits at maturity into a bifid structure – the carpophore is rarely entire or absent in the *Umbelliferae*. Each mericarp has a style that enlarges at the base forming a nectar secreting structure called a *stylopodium*, which is ± flat in *Bupleurum*. Each mericarp has five ridges (ribs or *costae*) separated by *valleculae* (or furrows if the ridges are prominent or winged; but the vallecular surface is ± convex in many species of *Bupleurum*). These ridges are primary ridges, as secondary ridges, also called vallecular ridges, are found in fruits of other genera (Arenas & García, 1993; Tutin, 1980).

Fruits in *Bupleurum* are usually oblong or oblong-elliptic or, more rarely, subglobose (e.g. *B. tenuissimum* and *B. semicompositum*). Mericarps are isodiametric, in cross-section (see Fig. 7.1A-B) or, sometimes, slightly laterally or dorsally compressed. Ridges are all equal, filiform or more or less prominent, rarely inconspicuous (e.g. *B. semicompositum*), but are in some cases winged (e.g. *B. angulosum* and *B. gibraltarium*) – the term 'narrowly' winged is more appropriate, as other umbelliferous genera that have very broad winged ridges are often simply described as 'winged' or having 'wings'. Fruit/mericarp length varies between 1 to 8(-11) mm. Surface is generally smooth, but rough fruits, tuberculate or papillose, are also found, e.g. those of *B. lancifolium* (Fig. 7.2A-B), *B. semicompositum* and *B. papillosum* DC. – the latter, an endemic of Turkey and

Iran (Snogerup, 1972), has remarkable inflated papillae that look like little whitish balloons on the surface of the fruit. Length of style normally varies between 0.15 and 1 mm; I have, however, found exceptionally long styles (up to 3 mm) in *B. album*, an endemic in Morocco.

Typical umbelliferous fruits show, in cross-section, a ± thin pericarp (divided into epicarp, mesocarp and endocarp) that surrounds an abundant endosperm (see e.g. Arenas & García, 1993).

A) Pericarp. The *epicarp* consists of a single layer of isodiametric or tangentially enlarged cells, with thick walls, covered with a cuticle of variable thickness. The *mesocarp* is formed mainly by parenchyma, and contains vascular bundles, fibres and secretory canals (oil ducts). The vascular bundles are located at the level of the (primary) ridges. The secretory canals can be found in the *valleculae*, and here are designated *vittae*, or in the ridges, externally to the vascular bundles. All these canals are schizogenous, i.e., they derive from the separation of cells and not from their lysis (Metcalfe & Chalk, 1983; Arenas & García, 1993). The *endocarp* is formed by a layer of tangentially enlarged cells, with ± thick walls.

B) Endosperm. The endosperm represents the major part of the fruit and is formed by polygonal cells rich in carbohydrates. The embryo is located in the middle of these tissue, in the upper part of the mericarp (closer to the stylopodium); the two cotyledons can be observed in sections made in the upper part of the fruit (see Fig. 7.2C).

The absence of sepals and the 'flat' stylopodium are considered important characters to distinguish *Bupleurum* from such possibly allied genera as *Hohenackeria* Fisch. & C.A.Mey (Mediterranean), *Nirarathamnos* Balf.f. (Socotra), *Heteromorpha* Cham. & Schltdl. (C & S Africa, Madagascar), and, *Anginon* Raf. (= *Rhyticarpus* Sond.) and *Hermas* L. from S Africa (Cauwet-Marc, 1976). Yet there are many others characters to distinguish these genera, like habit (*Heteromorpha* contains trees), leaf division (most of them have divided leaves), and inflorescence and other fruit characters, e.g.: *Hohenackeria* has simple umbels with sessile flowers, and the two mericarps remain fused after maturation; and *Heteromorpha* have unequal mericarps dorsally compressed and broadly winged.

Fruit anatomical characters in *Bupleurum*

Authors like Panelatti (1959), Cauwet (1976) and Arenas & García (1993) studied *Bupleurum* fruit anatomy. Panelatti (1959), in her anatomical study of leaves, stems and fruits of several Moroccan species of *Bupleurum*, considered that the most relevant fruit characters to distinguish the various species were: the presence/ absence of secretory canals in the ridges, and the number of vallecular and commissural *vittae*. Cauwet in her PhD thesis (1976, 1: 35) 'confirmed' Panelatti's opinion. However, Arenas & García (1976), in their study of the fruit of Iberian and Balearic *Umbelliferae* (subfamily *Apioideae*), used a larger number of anatomical characters to distinguish *Bupleurum* species (see their key to identification):

a) *Fruit surface ornamentation* [a macroscopic character, but still relevant in section as a distinguishing feature; Panelatti (1959) did not study species with rough fruits; and the species that most concerned Cauwet (1976) – her subgenus *Tenoria* – all had smooth fruits].

b) *Presence/ absence of vittae* [*vittae* were always present in the taxa studied by Panelatti (1959) and Cauwet (1976)].

c) *Number of vittae per vallecula.*

d) *Number of commissural vittae.*

e) *Size* [width] *of vittae.*

f) *Shape of the vittae.*

g) *Relative size of vittae when comparing to vallecular width* [area between the ridges].

h) *Presence of secretory canals in the ridges.*

i) *Size* [width] *of vascular bundles.*

j) *Endosperm shape* [of the side facing the commissure] straight/ concave.

k) *Prominence of ridges* [another macroscopic character, but still relevant in fruit section].

Arenas & García (1993) remarked that the most frequent numbers of *vittae* in all the taxa they studied in subfamily *Apioideae* (*Bupleurum* included), were one *vitta* per vallecula and two in the commissure. They also discussed that absence of *vittae* could be regarded as a derived (apomorphic) character as it is a rare feature in the taxa studied.

7.2 Material & methods

Initial training in anatomical techniques was received at the Botany Department of the University of Coimbra (Portugal). The staining procedures used then were the "Carmin aluné et vert d'iode" or the "Carmino-Vert de Mirande" techniques, adapted from Dop & Gautié (1928). The stains used in these techniques are *alum carmine* and *acetic iodine green* (used separately in the first, and as mixture in the latter). The use of these stains results in the following tissue differentiation: *cellulosic-pectic* cell walls stain in red or pinkish-red; lignified walls in green or blue-green; and suberized and cutinized walls stain in a brownish yellow. This is a fast method to obtain permanent mounted sections; they can be obtained from fixed material in 24 hr. However, there are two important disadvantages: **1)** sections need to be more than 20-25 μm thick or the iodine green will be totally removed during dehydration with ethanol; and **2)** xylene (= xylol), a considerably toxic chemical, is used.

In Edinburgh, at the Royal Botanic Garden, the staining procedure first used was the *safranin-alcian blue*, after wax embedded fruits were sectioned in a rotary microtome. Safranin (red colour) is a general stain, i.e. stains indiscriminately, and the alcian blue acts as the counterstain, staining cellulosic-pectic walls in greenish-blue. However, this procedure is very time-consuming, in particular the wax embedding, and the stains do not allow differentiation between lignified, suberized and cutinized tissues as they all stain in red. Also, as safranin can be easily 'washed', xylem or other lignified tissues may appear poorly stained.

Considering that the **Mirande's technique** is a fast method that can produce satisfactory results, it was chosen as the staining procedure to be used with some modifications, in particular to improve safety. The Mirande's technique is a relatively old procedure; Mirande described it in his thesis in 1900, but his was only a modified protocol of a staining technique already in use (Dop & Gautié, 1928). This technique has been used in many anatomical works, in particular by French botanists – Panelatti (1959) and Cauwet (1976) used it in their anatomical research in *Bupleurum*, although neither of them described the protocol they used.

The following is the protocol used in the present work:

Materials:

Stains:

Alum carmine: 100 ml of *distilled water*, 4 g of *potassium alum* (= aluminum potassium sulfate dodecahydrate – Sigma A-7167), 1 g of *carmine* (C.I. 75470; Natural Red 4 – Sigma C-1022), and a few crystals of *thymol* (5-methyl-2-isopropylphenol – Sigma T-0501).

Mix in an erlenmeyer the distilled water, potassium alum and carmine and heat the mixture on a hot plate stirrer (place a magnetic stirring-bar or 'flea' inside the flask). Simmer the mixture for c. 20 min or until carmine is dissolved. Leave to cool to room temperature and then filter. Add 3-5 crystals of thymol to the solution (thymol acts as a disinfectant to avoid fungal development in the stain). (*)

Acetic iodine green: 100 ml of distilled water, 1 g of iodine green (C.I. 42556 - DIFCO 8255), and 2-3 drops of acetic acid.

Dissolve the iodine green in the distilled water with the drops of acetic acid, and filter. (*) – Be very careful when opening the container of the iodine green; it is a very light powder that will spread everywhere and will be easily inhaled!

(*) Both stain solutions can be kept for many months (even more than one year), but need to be filtered before use.

Other material:

- ethanol (70%, 90% and absolute)
- bleach (5-10% solution of sodium hypochlorite)
- acetic water (10% glacial acetic acid)
- xylol (= xylene)
- 10% glycerin (= glycerol) – [i.e. 10% glycerin + 90% distilled water]
- euparal or Canada balsam
- small glass Petri dishes (c. 5 cm diameter) – the round cover of a staining jar may be used instead [do not use plastic, as it will be dissolved by xylol]
- microscope slides
- glass coverslips
- pipettes

- filter paper
- paper tissue (laboratory wipes)
- latex gloves
- scalpel & blades
- tweezers
- spatulated needle
- fine brush (soft hair)
- test sieve (with frame and mesh of stainless steel, aperture 32 – Endecotts Ltd., London) or a small piece of wire mesh (c. 3 cm^2) [if using the piece of mesh, give it a slightly concave shape and roll up its borders with a pair of small pliers]

Equipment:

- sliding microtome (Reichert, Austria)
- DC power unit (Hanovia Ltd.) and freezing stage
- stereoscopic microscope
- compound microscope
- fume bench system
- drying oven or hot plate

Method:

1) For fixation, place fruits (or other plant material) in ethanol 70% for 24 hrs. Fruits of umbellifers are fairly dry specimens, but other material, especially if succulent, will require a longer time for fixation. In this case, plant material should be first placed in 90% ethanol for 24 hrs, and then in 70% ethanol for 2-3 days (ethanol may need to be recharged).

2) Sectioning using the freezing microtome (a normal sliding microtome with attached freezing stage):

- Turn water on, switch on power unit and set power to 5.5 A.

- Build a frozen base for specimen with drops of 10% glycerin (a flat and smooth base is important or the ice will break easily).

- Place specimen in the centre of the ice base (cut the base of the fruit to allow to stand up) and slowly, drop by drop, built a tower of ice with broad base around the specimen.

- Place blade in the microtome, and brush it with a little of the glycerin water (10% glycerin) to make it more adherent to the sections. Adjust level of the stage that

supports the specimen in relation to the blade. Start sectioning (30-50 μm thick). Collect each section from the surface of the blade with a fine brush, and place it in a small Petri dish with distilled water.

- Check the first sections under a compound microscope to adjust thickness of cut – some material may be too soft for a very thin section.

After finishing sectioning, remove the blade from the microtome and keep it in a safe place (e.g. inside a Petri dish).

3) Drain the water from the Petri dish with the sections using the test sieve (or the piece of wire mesh) to prevent loss of sections (use the sieve or mesh to retain the sections when changing solutions). Due to superficial tension of the water, some may be retained by the mesh (it will depend of its aperture size). If this happens, soak the remaining water, from the other side of the mesh, with some paper tissue.

After draining the solution, some sections will stay in the Petri dish, but most of them will be on the mesh. Pour the following solution (in this case the bleach) through the mesh to put the sections back to the dish. If some sections still stay on the mesh, they can be removed, very carefully, with the help of a fine brush or a spatulated needle (do not use tweezers to handle the sections!). If using the wire mesh, sections may be put back to the dish by tapping the mesh with the back of a dissection needle (or similar object).

From now on work in a fume bench (recommended, but not strictly required) and use latex gloves to protect the skin (several of chemical used are irritant).

4) Leave sections in bleach for c. 10 min to destroy cell contents (for bleach concentration ≥ 5% leave for up to 25 min). Examine sections under a stereoscopic microscope to check when sections are clear – cells become gradually transparent as its contents are cleared.

5) Drain the bleach and pour the acetic water into the Petri dish. Leave the sections to wash for c. 5 min.

6) Remove acetic water and wash the sections twice in distilled water (5 min each).

7) Staining: Replace the water with the following stain mixture: 9/10 parts of *alum carmine* and 1/10 parts of *acetic iodine green*. This mixture should be prepared

only before use, but it can be used for up to 2-3 days – in this case, it will need to be refiltered [If using the stain mixture go to step 8].

Sometimes the stains will work more efficiently if they are used separately. In this case: **a)** Place sections in *acetic iodine green* for 5-10 sec. **b)** Remove stain and place material in 90% ethanol for 5-10 min (differentiation). **c)** Wash in distilled water for 5 min. **d)** Place sections in *alum carmine* for 5-10 min. **e)** Wash in distilled water for 5 min [If using the stains separately go to step 10].

8) Drain the stain mixture and place the sections in 90% ethanol for 5-10 min (differentiation). Examine sections under the microscope to check differentiation progress – the excess of acetic green iodine is slowly removed by the ethanol, if too much of this stain is left, cellulosic-pectic tissues will not be clearly shown.

9) Stop differentiation by placing the sections in water (c. 5 min).

10) Dehydration: Replace water by absolute ethanol (from now on, the wire mesh or sieve should not be used to avoid contact of the sections with water remaining in the mesh). Renew the absolute ethanol twice (5 min each). Use a tissue to dry any liquid left in the Petri dish – be careful, do not 'absorb' the sections!

11) Replace the absolute ethanol by a mixture of xylol and absolute ethanol (1:1), and leave for at least 5 min.

12) Drain the xylol/ethanol mixture and pour xylol (100%) into the Petri dish. At this point, if some whitish ('milky') emulsion is seen, it will mean that the sections are not completely dehydrated and that they should be put back into absolute ethanol to complete dehydration.

Renew xylol twice and leave for at least one hour, but preferably overnight. It is important that no ethanol is left in the sections. If ethanol is still present after mounting, the colour in the sections (especially the green) will fade with time.

13) To mount the sections, move the stereoscopic microscope to a fume bench – xylol fumes are fairly toxic. Examine the sections, that are inside the dish with xylol, under the microscope to mount only the best ones. Place a drop of euparal (or Canada balsam) on a microscope slide. Using a spatulated needle, move one section at the time and place it in the euparal drop – the number of sections per slide will depend on the size of the sections. After 1-6 sections have been moved to the

slide, place on top, very carefully, a coverslip, avoiding large bubbles of air (the minute bubbles tend to disappear during the drying process). Put a small weight on top of the coverslip – be careful to check that no excess of euparal will stick to the weight. The excess of euparal, in the borders of the coverslip, can be removed, with the help of a scalpel and xylol, after the sections are completely dry.

For safety (even if working in the fume bench) reduce the time of exposure to xylol fumes: do not mount specimens for longer than 1 hr ½. Mounting is the part of the technique where one is in more direct contact with xylol fumes as sections need to be selected and 'caught' from the xylol using the spatulated needle.

14) Label the slides and place them on a drying oven or heated surface for 2-3 weeks. The slides can be left to dry out at room temperature, however it is strongly recommended to dry the slides as soon as possible as changes of temperature will affect the quality of the sections. Low temperatures cause contraction of the mountant (euparal or Canada balsam), if not dry and rigid yet, and the sections will be distorted.

From the moment that the sections are mounted, they can be examined (photographed, etc) under the microscope. But care must be taken if the slides are not dry as the sections will be distorted if the coverslip is moved.

7.3 Results & discussion

Fruit material of the following 8 species was studied. For information on the accessions see Appendix II.

Taxon	Acc. Nos
B. acutifolium	228, 262
B. canescens var. *handiense*	28
B. fruticosum	5, 7
B. lancifolium	45, 73-76
B. longifolium	31
B. rigidum subsp. *paniculatum*	234
B. rotundifolium	4
B. salicifolium	29

Despite the fact that the staining technique used is relatively fast, it was only possible to study a small number of taxa within the time available for anatomical work. Some difficulties were found in obtaining the stains, in particular the iodine green – it has been taken out of the DIFCO catalogue in Britain for not being requested in recent years. Nevertheless, it was still possible to make some improvements in the technique, and to obtain some additional data relating fruit anatomical characters.

A) The technique

The use of the freezing microtome represented a significant improvement in the quality of the sections obtained. Previously, at the Departamento de Botânica, Universidade de Coimbra, cork or *Sambucus* pith were used to support the specimen during sectioning with satisfactory results. But the ice is a strong and more homogeneous support that completely surrounds the specimen, and because the ice melts when cutting, the sections are easily seen on the surface of the blade (no pieces of cork or pith are left, only drops of water).

Because of its toxicity by simple inhalation, the use of xylol (= xylene) has been avoided in modern anatomical protocols. However, I do not know any less toxic substance with equivalent chemical properties to substitute the use of xylol in the Mirande's technique. But, if work is carried out in a fume bench, as is here recommended, inhalation of toxic fumes would be minimise and the safety of the procedure greatly improved.

B) Fruit anatomical characters in *Bupleurum*

Within the species here studied it was possible to observe the sort of variation in fruit anatomical characters that have been recorded in the genus:

Bupleurum lancifolium (Fig. 7.2) and *B. rotundifolium* are example of species without *vittae* or other secretory canals (the two species are very closely related). The following species have one *vitta* per vallecule and two *vittae* in the commissure: *B. acutifolium* (Fig. 7.1A), *B. canescens* var. *handiense*, *B. fruticosum* (Fig. 7.3A), *B. rigidum* subsp. *paniculatum* and *B. salicifolium* (Fig. 7.1B). Of the species

studied, only *B. longifolium* has a larger number of *vittae*: 3-4 *vittae* per vallecula (see Fig. 7.3C), and 4 *vittae* in the commissure.

Secretory canals in the ridges (located externally to the vascular bundles) were seen in *B. canescens* var. *handiense* (Fig. 7.1C), *B. fruticosum* (Fig. 7.1D), and *B. salicifolium* (Fig. 7.1B). Although not seen in my fruit sections, Panelatti (1959) and Arenas & García (1993) observed small secretory canals in *B. rigidum*. A larger sample needs to be studied to verify if these canals may be present or absent in a single taxon.

Commissural face of the endosperm is clearly concave in several of the taxa studied e.g. *B. lancifolium* (see Fig. 7.2B) and *B. rotundifolium*, while in others seem to be ± flat (see Arenas & García, 1993). However, the shape of the endosperm varies depending of the level were the section is made and, therefore, this character do not seem to have great taxonomic value.

Bupleurum salicifolium and *B. canescens* var. *handiense* are closely related taxa, which have been confirmed by the ITS sequences (see sections 9.3-9.4, and also chapter 10), and the only difference observed in their fruits was that the former has larger *vittae* (each *vitta* is almost as wide as the vallecule – see Fig. 7.1B). However, more samples need to be studied to confirm this small difference between these taxa.

A fruit sample from each of the two populations of *B. acutifolium* (Spanish - Acc. No 262; and Portuguese - Acc. No 228) was examined, and the only (clear) difference found was the size of the vascular bundles, which were proportionally larger in Acc. No 228. However, more samples need to be studied before evaluating the relevance of this small difference – ITS sequences of the two populations of *B. acutifolium* were found to differ considerably, which has raised doubts about delimitation of this taxon (see section 9.4).

Most of the characters indicated by Arenas & García (1993) – see introduction of this chapter – seem useful in distinguishing the species of *Bupleurum*. However, the shape of the *vittae* could be an ambiguous character related with state of maturation of the fruit or even an artefact of the technique (ethanol may cause contraction of the sections, obviously altering the general shape of the structures).

In my investigation, I noticed a character not previously recorded: the relative position of xylem and phloem in the vascular bundles. Arenas & García (1993) and

other authors only indicated the general shape of vascular bundle without describing the position of the tissues. Panelatti (1959) did not discuss this character, but in her drawings she distinguished the xylem and phloem in the vascular bundles.

It seems that in most of the species studied, phloem is located externally to xylem (see Fig. 7.1C; and also in the drawings of fruit sections in Panelatti, 1959). However, two of the species I studied, *B. fruticosum* and *B. rigidum*, have the xylem placed between two small areas of phloem (see Fig. 7.1D). This is indeed a very interesting character, especially because the molecular data (ITS sequences – see chapter 9) strongly supports the two taxa being closely related, despite being different morphologically (see chapter 10 & 11 for further discussion). Panelatti (1959) studied the fruit of *B. rigidum*, but her diagram of its fruit (see Tab. 19, fig. 3) suggests that the phloem is located externally to xylem; possibly, she did not notice the different position of the tissues in this species.

7.4 Further work

The taxonomic value of characters like the size of *vittae* and presence/ absence of secretory canals in the ridges is not yet clear. In Arenas & García (1993), size of *vittae* seemed clearly distinct in some species, but the authors also registered some degree of variation. It is not known how the environmental conditions affect the development of these secretory structures.

The molecular evidence strongly suggest that the genus should be divided into two major groups (two new subgenera proposed in this work). So far one fruit character (relative position of xylem and phloem in the vascular bundles) seems to differ between members of these subgenera. However, it is necessary to confirm these findings with the study of more samples, in particular from *B. rigidum* (two different accessions were studied for *B. fruticosum*). It is also necessary to study many more taxa in the genus to see if this character has any taxonomic significance. It would be especially interesting to study the other members of the new subgenus *Penninervia* (see chapter 10), to see if any of them share the peculiar position of phloem in relation to xylem that was found in *B. fruticosum* and *B. rigidum*.

Bupleurum rigidum is morphologically very distinct from the other members of subgenus *Penninervia*, but the study of the leaf and stem anatomy may provide characters supporting the relatively close relationship of these taxa.

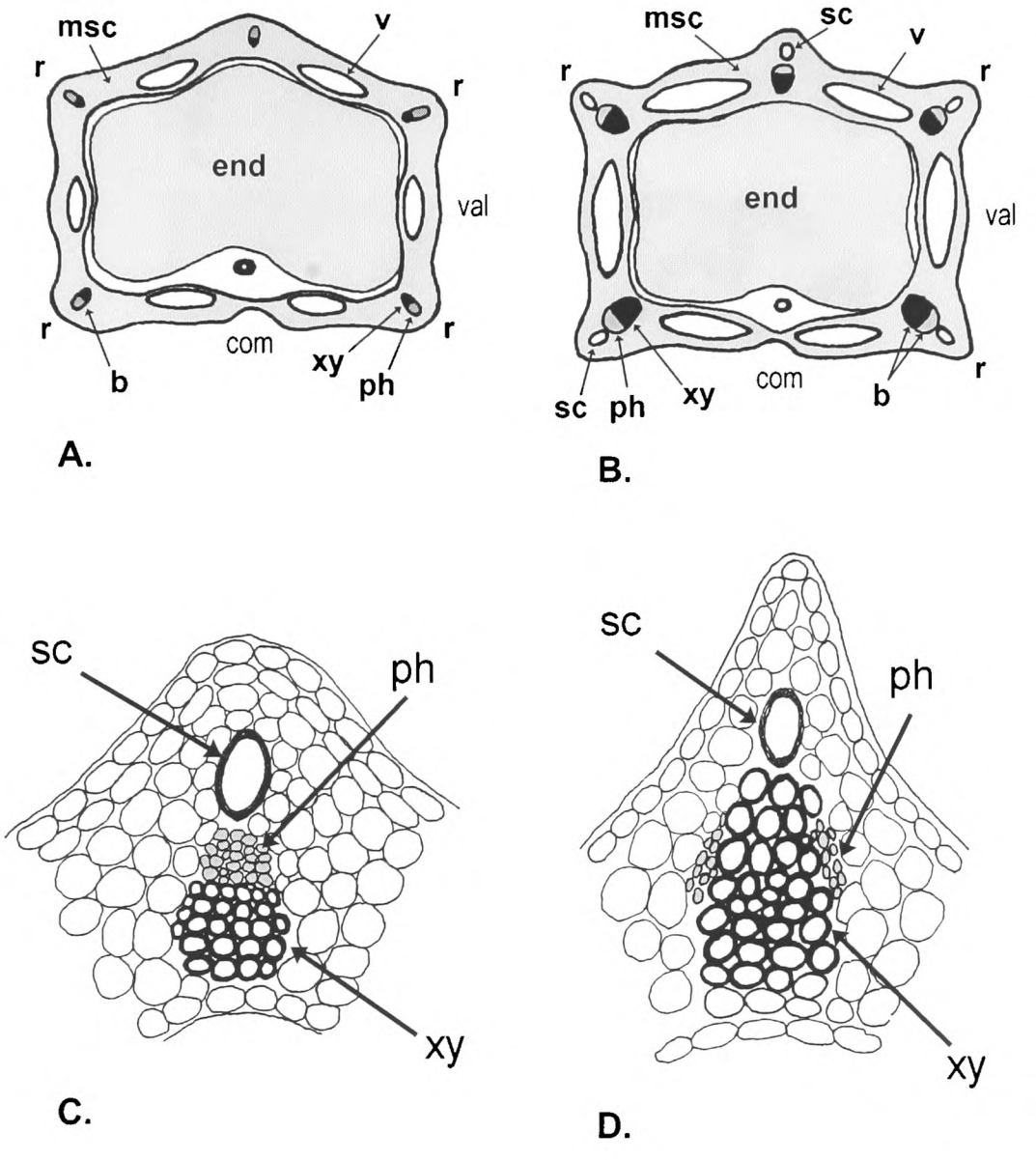
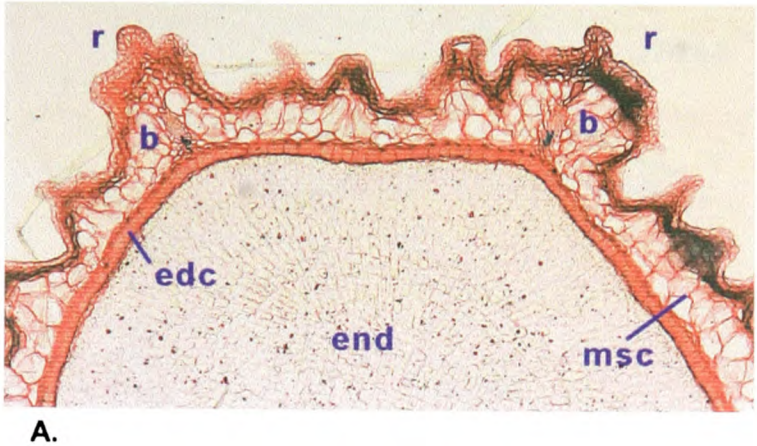
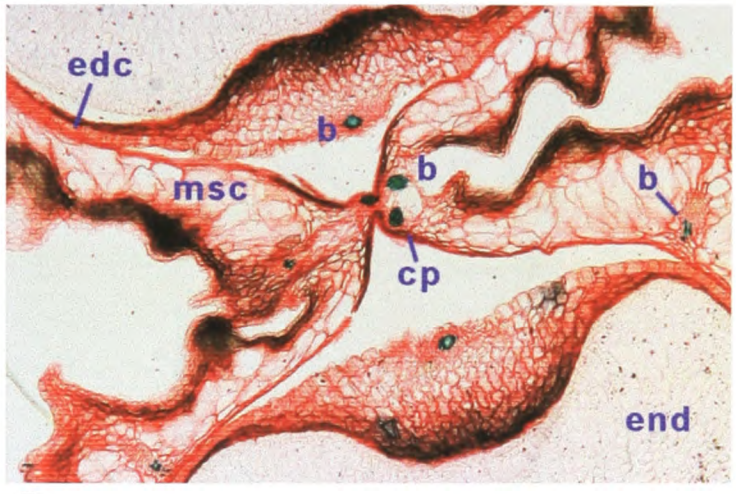


Fig. 7.1 - Diagrams of fruit sections in *Bupleurum*. A. *B. acutifolium* (Acc. No 262) - 45X. **B.** *B. salicifolium* (Acc. No 29) - 45X. **C-D.** Detail of mericarp sections - ridges (175X). **C.** *B. canescens* var. *handiense* (Acc. No 28). **D.** *B. fruticosum* (Acc. No 5). **end =** endosperm; **msc =** mesocarp; **v** = *vittae* or oil ducts; **sc** = secretory canals; **b** = vascular bundles; **ph** = phloem; **xy** = xylem; **com** = commissural face; **val** = vallecular face; **r** = ridges.



A.



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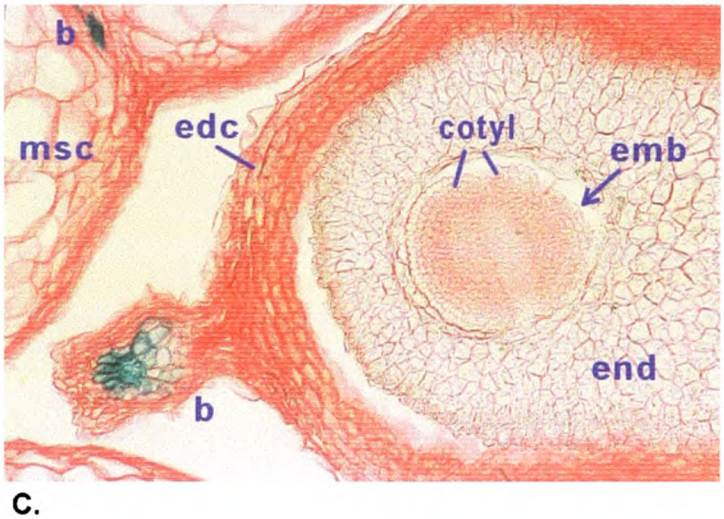
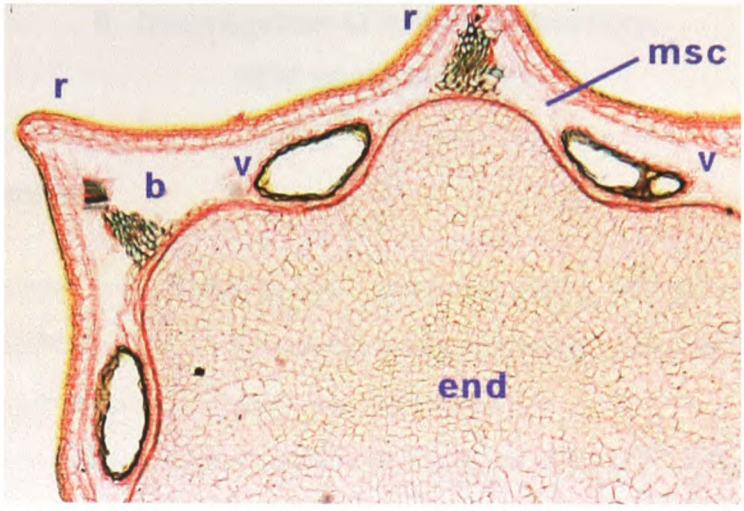
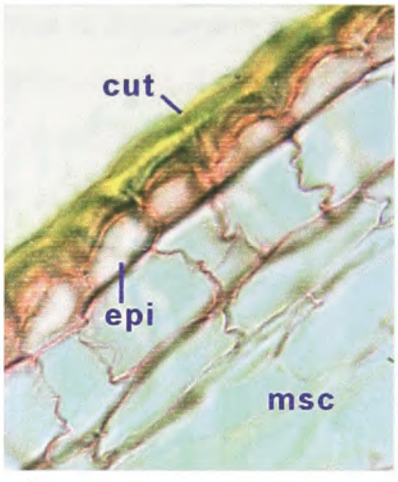


C.

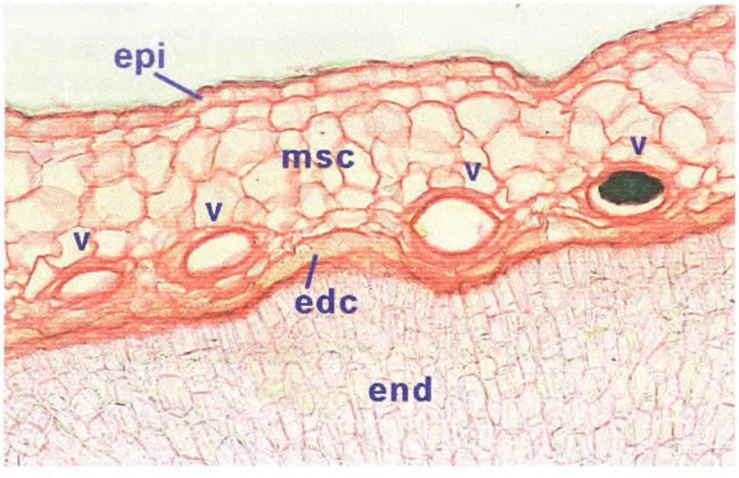
Fig. 7.2 - Transverse fruit sections of *Bupleurum lancifolium* (Acc. No 45). **A.** Partial view of mericarp (mid-section of fruit); notice the rough surface, the vascular bundles (**b**) in the ridges (**r**), and that *vittae* (oil ducts) are absent (X54). **B.** Joining area of the two mericarps (mid-section) (X54). **C.** Section at the upper part of the fruit; notice the embryo (**emb**) and its two cotyledons (**cotyl**) (X130). **cp** = carpophore; **edc** = endocarp; **end** = endosperm; **msc** = mesocarp.



A.



B.



C.

Fig. 7.3 - Fruit sections of *Bupleurum*. A-B. – *B. fruticosum* (Acc. No 5). **A.** Partial view of mericarp (mid-section of fruit); notice the large *vittae* (**v**), one per *vallecula* (X54). **B.** Epidermis (**epi**) and underlying layers of cells; notice thick cuticle (**cut**) (X230). **C. *B. longifolium*** (Acc. No 31). Partial view of mericarp; notice the 4 *vittae* per *vallecula* (X130). **b** = vascular bundle; **edc** = endocarp; **end** = endosperm; **msc** = mesocarp; **r** = ridges.

8. Investigation of micromorphology: SEM study of fruits

8.1 Introduction

Bupleurum fruit material was examined using the scanning electron microscope (SEM) to look for potentially useful characters of the epidermal surface. For description of the fruit in *Bupleurum*, see section 7.1.

As far as I know, there is no published account of a study of *Bupleurum* fruits using SEM. Cauwet (1976, 1: 32) briefly mentioned in her PhD thesis that she used the SEM to study fruit surface in *Bupleurum*, but remarked that all of her samples showed great uniformity. Unfortunately, she did not indicate the species she studied, number of samples or the characters she thought were uniform. I tried to contact Dr Cauwet to her address at the University of Perpignan, France; but I was unsuccessful.

8.2 Material & methods

Material:

- fruits (mericarps).
- aluminium stubs (12.5 mm diameter).
- double-sided adhesive carbon discs.
- silver dag (Acherson 915 silver dag, Acherson colloids company, Prince Rock, Plymouth).
- gold coater - Emscope SC 500A (Agar Scientific - 66A Cambridge Road, Stansted)
- SEM microscope - Microscope Cambridge Stereoscan 250 (L.E.O. Electron Microscope, Cambridge).
- 35 mm film - Kodak TMax. 100.

Methods:

- Stick a carbon disc to each stub, and place 2 or more mericarps on each of them (label the back of the stub indicating accession number of sample).

- To increase electron conductivity, with a fine brush apply a small amount of silver dag to the area of contact of the fruit with the stub, and to borders and top of fruits – for the smaller fruits like those of *B. tenuissimum* and *B. semicompositum* (or

pollen and other small or flat structures) the silver dag is not necessary. Notice that epidermal surface will be hidden by the silver dag, so use only the amount strictly needed (the SEM technician can advice you on this).

Material will be ready for examination after being coated with a layer of gold a few nanometres thick (there is a slight variation on the thickness of the layer deposited by the gold coater, but this is only relevant in studies using extremely high magnifications). Voltage used at the SEM was 7-10 KV (kilovolts).

Concerning provenance of material, mericarps used were either: 1) collected by myself in the wild or from cultivated plants; 2) spare from successfully germinated plants; 3) sampled from herbarium specimens; or 4) received from other Botanical Gardens but only used after comparing with identified herbarium material.

The following table lists the taxa and accessions studied for *Bupleurum* (16 species) and other genera: *Anginon* [= *Rhyticarpus*], *Heteromorpha*, *Hohenackeria* and *Nirarathamnos*.

Taxon	Acc. Nos
Bupleurum angulosum	174
B. falcatum	118
B. fruticosum	5, 119
B. gerardii	69
B. gibraltarium	183
B. lancifolium	45
B. longifolium	165
B. oligactis [= *B. atlanticum*]	92
B. praealtum	3, 112
B. ranunculoides	181
B. rigidum subsp. *rigidum*	190
B. rigidum subsp. *paniculatum*	86, 87
B. rotundifolium	4, 171
B. salicifolium	199, 212
B. semicompositum	188, 189
B. stellatum	133
B. tenuissimum	90, 187
Anginon difforme	191-193
Heteromorpha trifoliata	197
H. transvaalensis	204
Hohenackeria exscapa	194
Nirarathamnos asarifolius	195

8.3 Results & discussion

The figures 8.1 to 8.4 represents a selection of SEM photographs of fruits of *Bupleurum* and the other genera studied.

Only a few characters of the fruit surface in *Bupleurum* seem to show some significant variation:

1) Surface shape of epidermal cells: concave or convex.

Bupleurum rotundifolium fruits have a concave epidermal cell surface (Fig. 8.1D), this was confirmed by my own anatomical study. A close species, *B. lancifolium* seems to share this character, but interpretation of the SEM images is more difficult because of the rough ornamentation of its fruits (Fig. 8.1A & B). *Bupleurum gerardii*, *B. praealtum* (Fig. 8.2E & F), *B. ranunculoides*, *B. salicifolium* (Fig. E), *B. semicompositum* (Fig. 8.2D), and *B. tenuissimum* (Fig. 8.2A-C), show a clearly convex epidermal cell surface; in *B. stellatum* it is slightly convex. *Bupleurum rigidum* (Fig. 8.3E) seems to have a more or less flat epidermal surface, but fruit sections confirmed that a thick cuticle hides the real shape of the epidermal cells, which is also convex. The same situation occurs in *B. fruticosum* (Fig. 7.3B, in the previous chapter) and *B. gibraltarium* (Fig. 8.3F) where a very thick cuticle covers epidermal cells with an evident convex surface. *Bupleurum angulosum*, *B. falcatum* and *B. oligactis* also show a 'flat' surface, but further anatomical study is necessary for these species.

The concave epidermal cell surface may be restricted to the '*Perfoliata*' group (see chapter 10); fruit anatomy confirmed the feature for these taxa. However, the SEM image will not be conclusive if surface appears to be flat as a thick cuticle may be hiding the real shape of the epidermal cells.

It could be argued that the external shape of epidermal cells changes during drying of fruits; but all fruit material studied was fully dry. In addition, mericarps were confirmed fully developed for some of the samples, as germination of same accessions (see chapter 5) was successful (e.g. *B. rotundifolium* Acc. No 171 - *concave cell surface*; and *B. gerardii* Acc. No 69 - *convex cell surface*).

2) Epicuticular wax type: granules, rods, filaments, plates, scales, aggregate coatings, or mixed coatings – see Metcalfe & Chalk (1979, p. 158-162).

Wax coating in *B. angulosum, B. lancifolium, B. rotundifolium* (Fig. 8.1D), *B. salicifolium, B. semicompositum* and *B. tenuissimum* (Fig. 8.3A) is mainly formed by granules or aggregations of these, but some material also presents scales, sometimes just visible using very high magnifications (> 4500X) (see *B. rotundifolium*, Fig. 8.3C). Scales are the main wax coating in *B. gerardii, B. praealtum* (Fig. 8.3B) and *B. rigidum*. A mixed coating with granules, rods, filaments and scales is found in *B. falcatum*. *Bupleurum fruticosum* fruits have clear wax plates (Fig. 8.3D), and also small scales that are embedded in the cuticle.

Amounts of wax vary quite greatly in different areas of the same fruit or in different fruits of the same species; normally a larger amount of wax is found on the commissural face. The type of wax coating seems to be similar between closely related species such as *B. tenuissimum* and *B. semicompositum*, or *B. fruticosum* and *B. gibraltarium*. However, correlation between type of wax and morphological similarity seems to be poor, as very different species share similar type of wax.

3) Stomata location: sunk or superficial.

Stomata were not evident in most of the material; they were only clearly visible in some fruits of *B. rigidum* (Fig. 8.3E). In the fruits of *B. fruticosum* and *B. gibraltarium*, I found several look-alike canals (Fig. 8.3F) which, I later realised, were cavities left by the degeneration of sunken stomata – I have confirmed this observation in fruit sections of *B. fruticosum*.

However, if stomata location has any taxonomic value, we should study them in the organs where they are always present and clearly visible (i.e. leaves). In the fruit a comparative study is not possible as the stomata were too often not visible. In fruits, stomata function may be restricted to the early stages of flower/ fruit development, with only a marginal function, if any, in later stages.

8.4 Conclusions

The epidermis of *Bupleurum* (all organs) typically lacks structures of taxonomic interest, i.e. hairs, glands, etc. In other plant groups, study of fruit surface

has contributed important taxonomic characters. But, in *Bupleurum* fruits, there is little to study and the characters found do not appear to have diagnostic or systematic value. The following are the conclusions concerning the characters studied:

1) *Epidermal cell shape* may have some taxonomic value, but fruit anatomy is often necessary for accurate interpretation of the SEM images.

2) *Wax coating* is only visible using SEM, but, despite the variety of wax coatings found, very different species present very similar coatings, and considerable variation (amounts and wax type) is found in fruits of the same species, even within the same fruit. Cuticular ornamentation and wax coating have had diagnostic value in other plant groups (Metcalfe & Chalk, 1979, p. 143-147), however, at least in the fruits, wax coating has a limited taxonomic value in *Bupleurum*.

3) *Stomata* in the fruits are rare or difficult to see (not seen in most of those studied). Therefore, as it is not possible to make a systematic study of the various species, fruit stomata have only limited interest, e.g. in the understanding of the ecology of the species – sunk stomata are often an adaptation to dry environments.

The study of fruits of some of the genera (see Fig. 8.4) that have been associated with *Bupleurum*, only confirmed what the general morphology seems to indicate: there is no obvious character that supports a close relationship between *Bupleurum* and any of these genera. However, fruit surface seems to be clearly distinct between these genera, and the study of more species may prove that characters of fruit surface may have a diagnostic value at the generic level.

SEM study of *Bupleurum* fruits is not a promising field in the taxonomy of the genus, as there is only a small number of features to study and their variation is not consistent. Nevertheless, SEM study should not stop with fruits, as other organs, such as leaves and stems, may provide more useful information. But in any case, and giving the lack of superficial structures in *Bupleurum*, anatomy seems by far a more interesting and potentially useful area of study (see chapter 7). SEM should be used more to clarify particular problems, for instance, those that may arise from anatomical study.

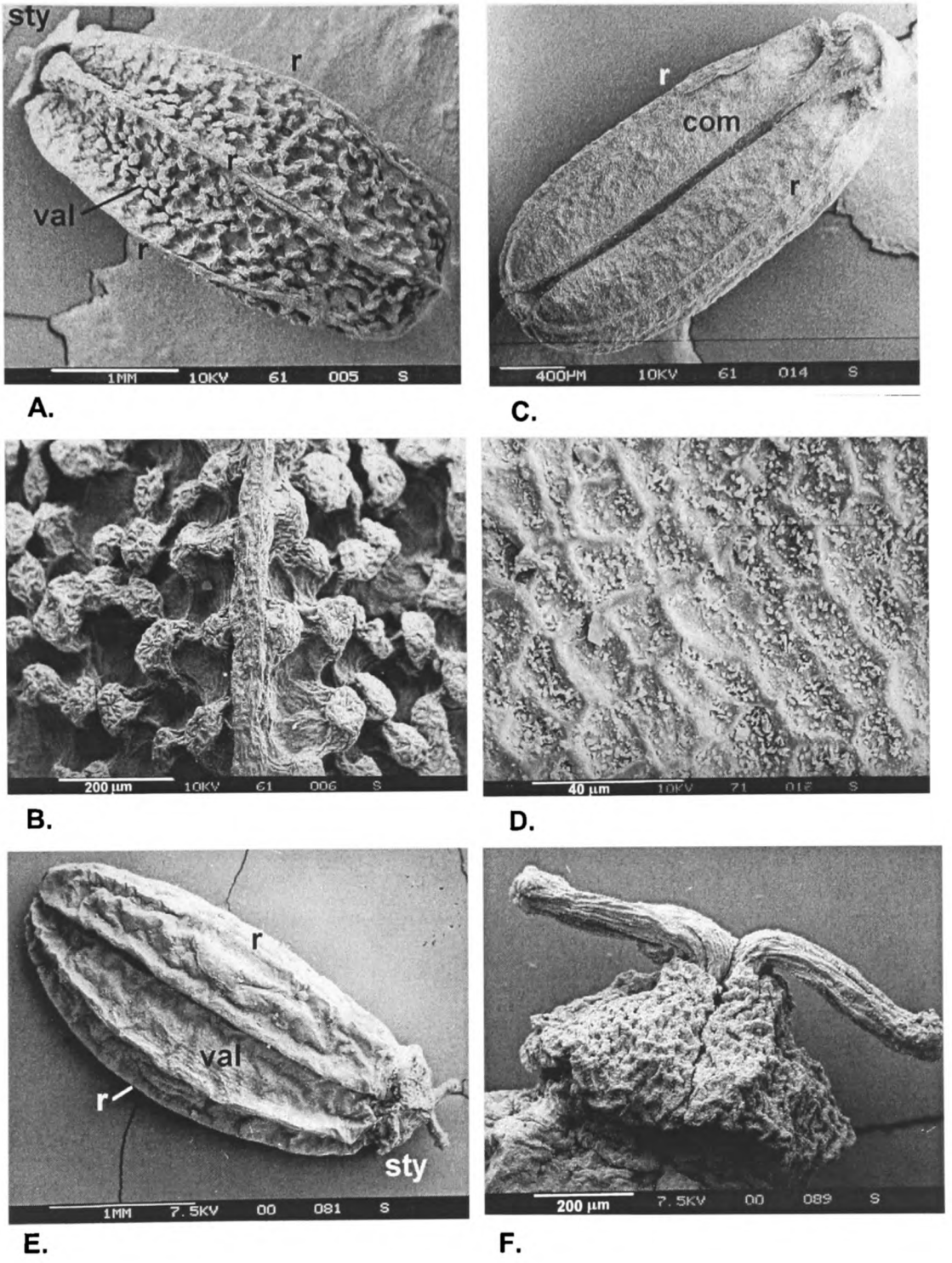


Fig. 8.1 – A-B. *Bupleurum lancifolium* (Acc. No 16) - dorsal view of mericarp; notice the ridges (**r**) and, between them, the *valleculae* (**val**). **C-D. *B. rotundifolium*** (Acc. No 4) - commissural view of mericarp; in **C.** notice the furrow left by the carpophore. **D.** surface of mericarp; notice concave epidermal cell surface. **E-F. *B. salicifolium*** (Acc. No 212). **E. -** Lateral view of the fruit (the two mericarps are still attached). **F. -** The stylopodium disk and the styles. **com =** commissural face; **sty =** stylopodium.

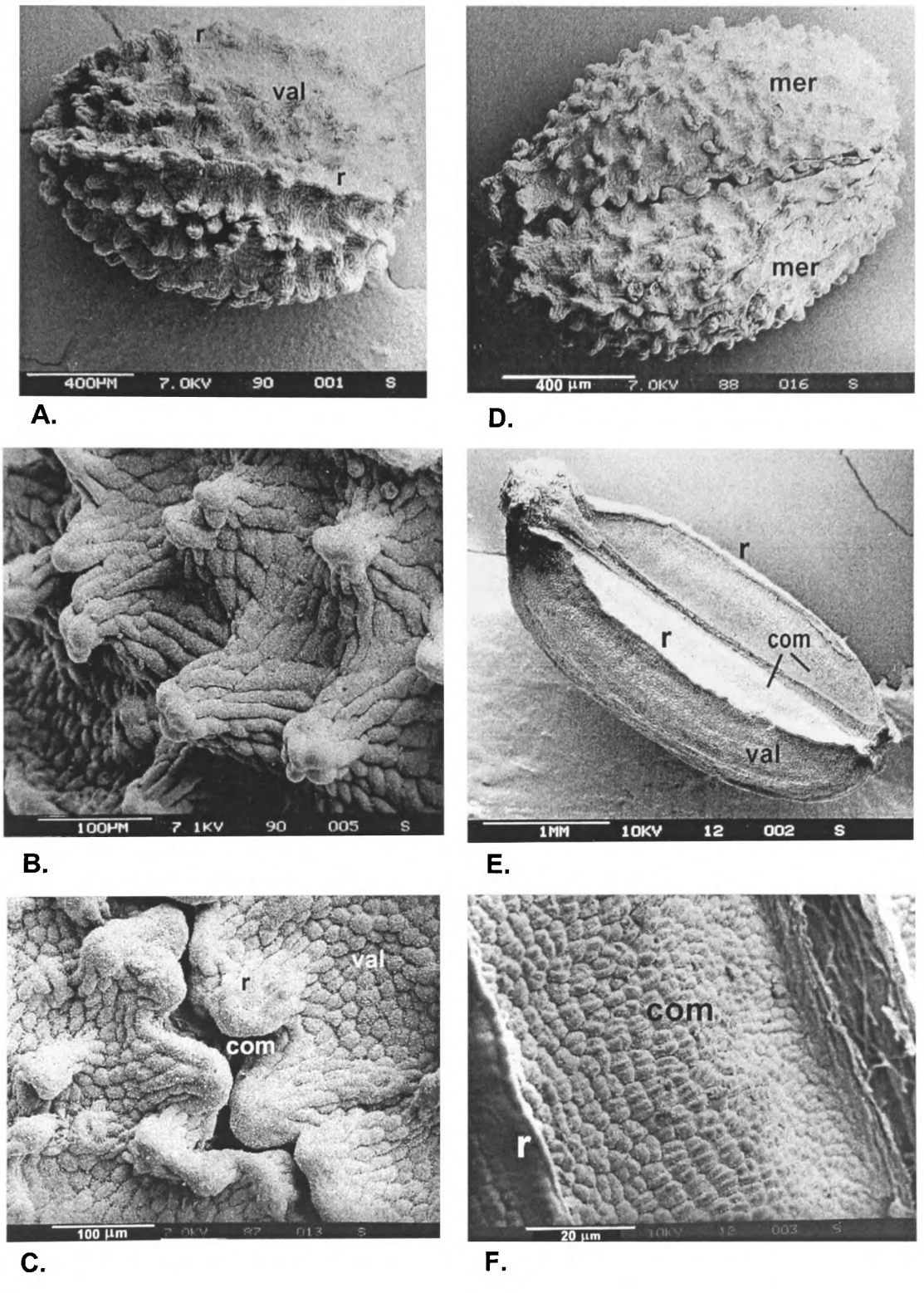


Fig. 8.2 – A-C. *Bupleurum tenuissimum*. A-B. - dorsal view of mericarp; notice the ridges (**r**) and *valleculae* (**val**) (Acc. No 90). **C. -** lateral view; notice the joining area (commissure - **com**) of the two mericarps (**mer**) (Acc. No 187). **D. *B. semicompositum*** (Acc. No 188) - lateral view of fruit; the two mericarps are still attached. **E-F. *B. praealtum*** (Acc. No 112) - view of mericarp. **F.** detail of commissural surface.

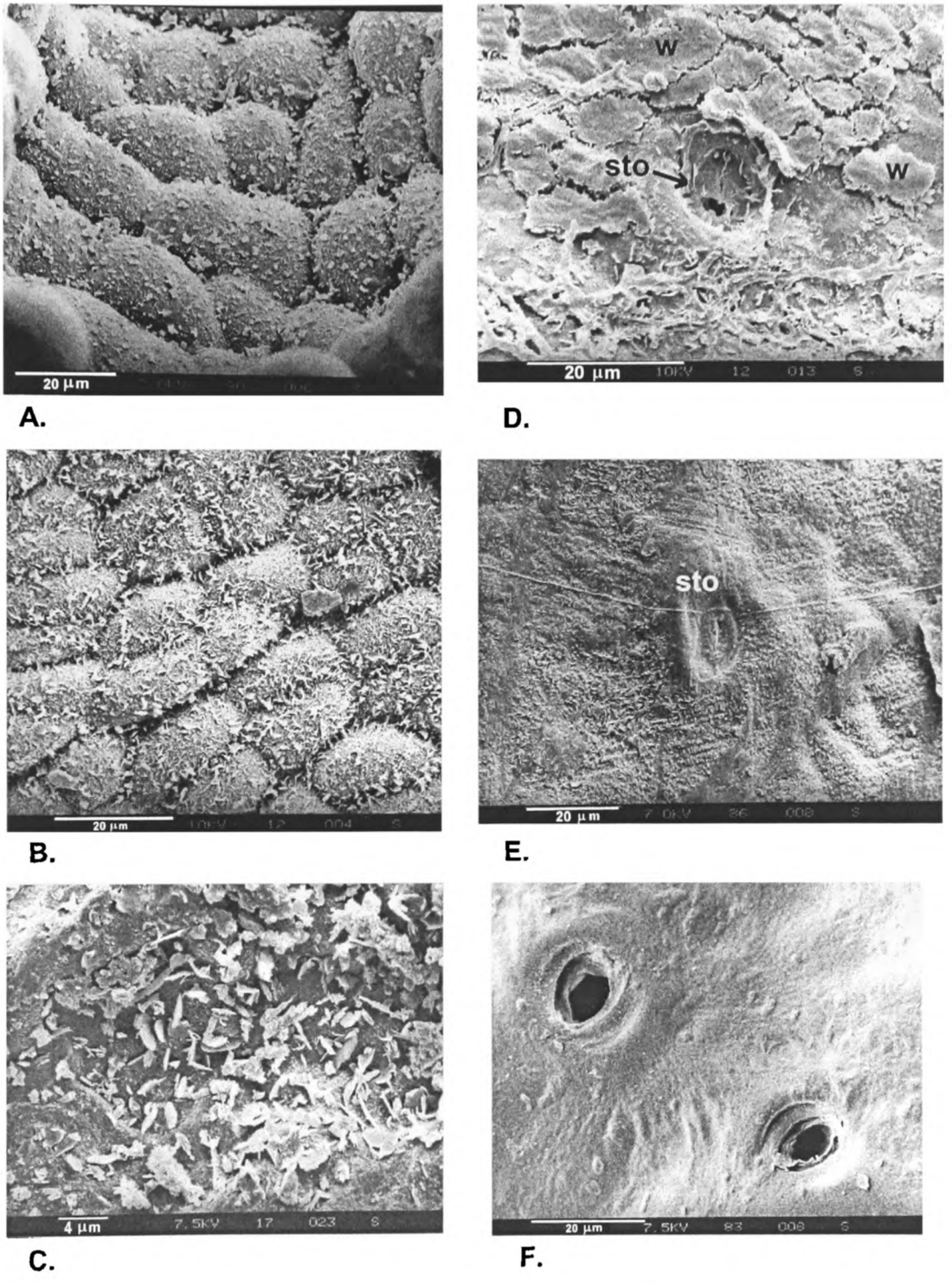


Fig. 8.3 – Fruit surface in *Bupleurum*. A. *B. tenuissimum* (Acc. No 90) - notice convex shape of epidermal cells and the granular epicuticular wax. **B. *B. praealtum*** (Acc. No 112) - notice the epicuticular wax (mainly scales). **C. *B. rotundifolium*** (Acc. No 4) - epicuticular wax (scales and granules, or aggregates). **D. *B. fruticosum*** (Acc. No 119) - notice the wax plates (**w**) and the stoma (**sto**). **E. *B. rigidum*** (Acc. No 86) - stoma. **F. *B. gibraltarium*** (Acc. No 183) - notice the cavities left by the degeneration of sunk stomata.

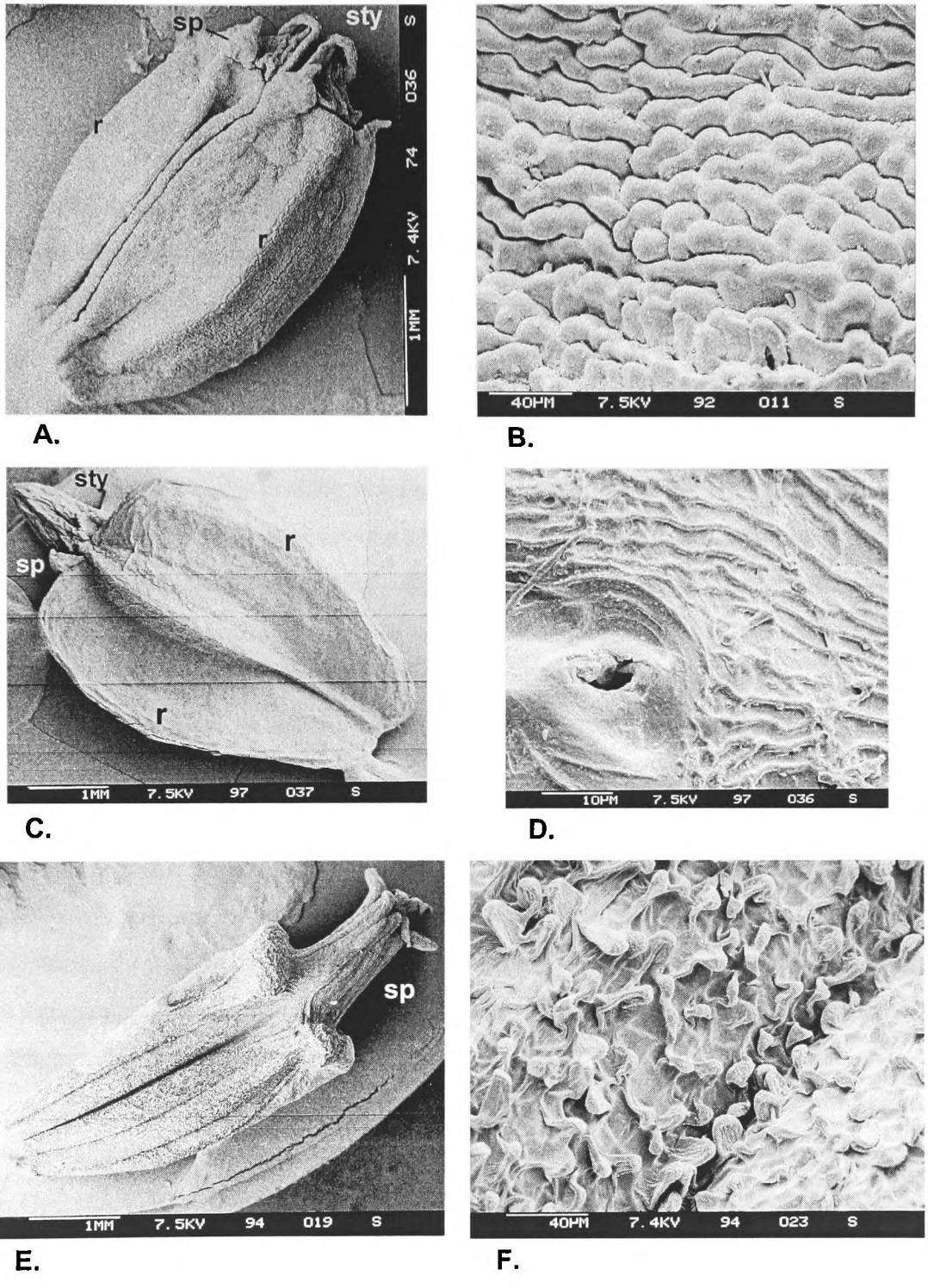


Fig. 8.4 – A-B. *Anginon difforme* (Acc. No 193). **A.** Lateral view of fruit; notice the presence of sepals (**sp**). **B.** Detail of fruit epiderm. **C-D. *Heteromorpha arborescens*** (Acc. No 197). **C.** Dorsal view of mericarp (dorsally flattened); notice the conic stylopodium (**sty**), the sepals, and the broadly winged lateral ridges (**r**). **D.** Detail of fruit surface; notice the cavity left after degeneration of a stoma. **E-F. *Hohenackeria exscapa*** (Acc. No 194). **E.** Lateral view of mericarp (carpophore is absent and mericarps remain fused at maturity); notice the conspicuous sepals. **F.** Detail of fruit surface.

9. Investigation of molecular biology

9.1 Introduction

Molecular phylogenetic studies have used data from two types of macromolecules: *proteins* and *nucleic acids* (mostly DNA). The study of macromolecules in taxonomy has several advantages over other chemical studies (e.g. secondary metabolites – see also section 3.6). DNA is the genetic material and has the highest content of information for systematic studies; proteins are still close sources, if indirect, of genetic variation (there is some loss of information as explained below – see protein sequencing). Metabolites represent variation many steps away from the genetic material, often being the products of complex biochemical pathways that need to be known for interpretation of data. Even then, very little reliable information can be inferred about the underlying genetic variation (Crawford, 1990, p. 13-17).

The diversity of methods that have been used in molecular systematics, especially in the last decade, and the theory behind the different approaches are intimidating, and newcomers to this research may feel totally overwhelmed (I certainly was). The increasing popularity of molecular systematics, in particular DNA studies, relies in the simple fact that, for the first time, taxonomists can gather genetic data (directly or inferred) using methodologies that only require a minimum of technical expertise, and can be cost-effective (still expensive, but the data obtained often justifies the investment).

The principles and methods (including detailed protocols) of molecular studies and their application in phylogenetic analysis are comprehensively discussed in *Molecular Systematics* of Hillis & Moritz (1990), in its second edition Hillis *et al.* (1996a), and also in *Molecular Tools for Screening Biodiversity* of Karp *et al.* (1998). Other useful references on molecular evolution and application of molecular data in phylogenetic studies are Avise (1994), Crawford (1990), Hollingsworth *et al.* (1999), Li (1997), Li & Graur (1991), Page & Holmes (1998), and Soltis *et al.* (1998).

Some karyological (cytogenetic) techniques, such as chromosome painting and banding, and *in situ* hybridization of nucleic acids probes to chromosomes,

provide data on molecular structure of chromosomes (see e.g. Sessions, 1996). However, as pointed out in section 3.8, karyological work requires living plants which are available only for a very small number of species in *Bupleurum*, and none for the more problematic taxa under study. For this reason, molecular cytogenetics are not discussed here.

The following section (9.1.1) is a summary review of the different techniques that have been more commonly used in molecular systematics, trying to point out their advantages and limitations. Different methods of data analysis are also briefly discussed (section 9.1.2).

9.1.1 Molecular techniques

A) Protein studies

1) Protein immunology: serology

The immunochemical studies of Nuttall, initiated at the end of the 19th century, can be regarded as the first studies of molecular phylogeny. Nuttall, working with various animal groups, showed that serological reactions were stronger for closely related species than for more distantly related taxa (see Li, 1997, p. 2 & 99; or Maxson & Maxson, 1990).

The principle of the serological technique lies on the specific **antigen–antibody** interaction. When a foreign substance, i.e. antigens (in this case, extract of plant proteins), is injected into an animal (usually rabbits or mice), there is an immunological response by the organism that results in the production of a special type of proteins, the *immunoglobulins* (antibodies). A few weeks after antigen has been injected, a blood sample is taken, and the antiserum (containing antibodies) produced is reacted with the original protein extract (‘**self-reaction**’ or control-reaction) and with extracts of other species (**cross-reaction**). The strength of the antigen-antibody reaction (precipitate formed) is measured. The assumption is that the degree of antibody-antigen reaction provides quantitative data on antigen similarity between different species. For discussion of serological techniques see e.g. Crawford (1990, p.191-222), Cristofolini (1980) and Maxson & Maxson (1990).

The specificity of the antigen-antibody reaction resides on surface features of parts of these molecules; these parts are designated **determinants** (or epitopes) in the antigen, and **antideterminants** (or paratopes) in the antibody. Each antigen has several determinants, from 5 or 6 up to a thousand per molecule (number depending on protein size). Several amino acids (5 to 10), contiguous or not, are involved in each determinant.

There are several limitations to the use and interpretation of serological data: **1)** Different animals need to be used, and even if they are reproduced by inbreeding to decrease genetic variation, there is always some degree of variation on the immunological response between different individuals; also the response of a single animal can vary over the time, with the possibility of becoming tolerant to certain antigens. **2)** Serological data are obtained by comparing two samples at a time: if antigens of species A and B are reacted with antibodies raised against a species C; we can infer the degree of similarity of A to C, and B to C, but nothing can be said about the similarity between A and B (antigens A and B may have different determinants in common with C). So, if many species are being compared the number of reactions needed will be very large, limiting the taxa that can possibly be studied. **3)** Antibodies recognize the surface shape of determinants and not real amino acid sequence; so, it is possible that underlying a similar shape there could be a very different sequence (convergent evolution). **4)** The reaction of antigen-antibody is often an average of the interaction in the different active sites. Antiserum is generally a complex mixture of antibodies of different types, each type specific for a particular determinant; if the affinity is very strong for a few of them, the amount of precipitate will be high, suggesting a similarity greater than real – [It is possible to prepare homogeneous populations of antibodies (*monoclonal antibodies*) that can specifically recognise single determinants; however, this is a very complex and expensive technique mainly used in immunological tests – see e.g. Weiler, 1986]. **5)** There are also ethical questions about the necessity of using animals, especially now that more trustworthy techniques are available in molecular systematics.

Serology has had a significant impact in taxonomy, and studies have been carried out in many plant families, including the *Umbelliferae* (e.g. Pickering & Fairbrothers, 1971; Shneyer *et al.*, 1992 & 1995). However, the technique is now

little used in phylogenetic studies as other methods provide more reliable and abundant data.

2) Protein electrophoresis and isozymes

Five out of 20 amino acids are electrically (positively or negatively) charged, and their total number determines the net charge of a protein. Shape and size also affect protein mobility. Some methods of electrophoresis separate proteins on basis of charge and size, while others separate only by charge. After proteins have been separated on gel (starch, polyacrylamide, cellulose acetate or agarose gels), the different bands are detected by histochemical staining (see Murphy *et al.*, 1996).

Although some studies have investigated the electrophoretic properties of non-enzymatic proteins (see e.g. Crawford, 1990, p. 30-50), the vast majority of studies have used **isozymes** (= isoenzymes), which are structurally different forms of enzymes with the same function. Isozymes can be produced by different loci or by different alleles of the same locus, the latter are termed **allozymes** (= alloenzymes).

The main assumption of studying proteins under an electrical field is that changes of mobility reflect differences in the amino acid sequence and are, therefore, genetically based. A second assumption is that enzyme expression is codominant, i.e. that all alleles are expressed, it is then possible to precisely distinguish homozygotes from heterozygotes. For correct interpretation of band pattern it is necessary to know the genetic controls and the distribution of enzymes in the cell and tissues. For detailed description of different methods that can be applied to isozyme electrophoresis see Murphy *et al.* (1996).

The main uses of isozymes are at the population level. They allow quantification of heterozygosity, evaluation of genetic diversity, help in delimitation of species, study of breeding structure and hybridization. Isozymes have also been used in phylogenetic studies, but their application is limited. The main limitations are: **1)** For some taxa, there is lack of variability and very few informative characters are found even when many loci are surveyed; and in the other extreme genetic divergence is such that no alleles/bands are shared, or bands shared are convergent (Murphy *et al.*, 1996). **2)** The genes encoding these enzymes represent a very small sample of structural genes as water-insoluble enzymes, or those that are bound to cell

structures are not included – [not forgetting that structural genes themselves are only a part of the whole genome]. **3)** Only nucleotide substitutions that affect electrophoretic mobility can be detected. **4)** There is the possibility that the same band represents two different alleles with identical mobility. **5)** Post-translational modifications that are not coded in the DNA may affect the conformation of the enzyme (secondary and tertiary structure), modifying its mobility – Müller-Starck (1998), Schaal & Learn (1988).

Nevertheless, isozymes are still widely used to investigate genetic variation in natural populations, mainly because the procedures are simple, the cost is low, and large amounts of data can be obtained.

3) Protein sequencing

During the 1970s and 1980s, several studies used protein sequences to infer phylogenetic relationships in plants, in particular, at higher levels of the classification (see Boulter, 1980; and Crawford, 1990, p. 171-190). However, there are several methodological limitations. First, not all proteins can be easily sequenced, they need to be: **a)** relatively small (≤ 100 amino acids) – sequencing of large proteins is a complex procedure that is not cost-effective (some studies opted to sequence only part of the protein after initial trials had determined that this selection will not bias the data); **b)** abundant in plant tissues; **c)** easily isolated and purified; **c)** homologous among the taxa compared [a requirement of data in any phylogenetic studies].

As a result, only a small number of plant proteins have been sequenced for taxonomical purposes, mainly: *cytochrome c* [component of the respiratory electron-transport chain of mitochondria]; *plastocyanin* and *ferredoxin* [components of the electron-transport chain of chloroplasts]; and the small subunit of *rubisco* [= ribulose bisphosphate carboxylase – the chloroplast enzyme that catalyses CO_2 fixation in the Calvin cycle] – see Boulter, 1980; and Crawford, 1990.

Some of the initial enthusiasm with protein sequencing was due to the fact that, at the time (1970s), it was the best available method to infer nucleic acids sequences. However, there is considerable loss of information in going from DNA to proteins. First, the sequence of the messenger RNA (mRNA) encoding a particular protein cannot reliably be inferred from the amino acid sequence (the genetic code is

degenerate – there are synonymous codons). Also, not all the DNA sequence that is transcribed is present in mature mRNA (certain parts are excised – the introns). At present, it is far easier and faster to sequence DNA directly than the proteins.

Not only there are technical difficulties in obtaining protein sequences, which obviously reduces the number of taxa that can be studied in a given time, the results also showed a high degree of convergence/parallelism in protein structure in the plant families studied (see e.g. Boulter, 1980, p. 237). The parallelism of structure is possibly due to functional constrains: the proteins sequenced have essential metabolic roles (respiration, photosynthesis), and many of the potential amino acid substitutions would affect the activity of the protein.

The data obtained from protein sequences failed to have the impact on plant taxonomy and phylogeny that was initially expected. Nevertheless, it raised many questions and stimulated much discussion about evaluation of different data sets, time of lineage divergence, and character homology at high levels of the classification (Crawford, 1990, p. 190).

B) DNA studies

1) DNA-DNA hybridization

This method relies on the double-stranded structure of DNA where the complementary strands are held together by hydrogen bonds between paired nucleotides. When DNA is heated up to c. 100°C the hydrogen bonds break and the two strands dissociate (DNA 'melts') – this temperature is not high enough to break the covalent bonds of DNA. Subsequent cooling allows reassociation of complementary strands forming **homoduplex** DNA (strands from the same sample of a particular species) or **heteroduplex** DNA (strands from different species/ taxa).

DNA-DNA hybridization involves a fairly complex methodology (for detailed protocols see Werman *et al.*, 1996), but is summarised in Appendix III(1).

DNA-DNA hybridization has been used at various levels of the classification in different plant families, including the *Umbelliferae* (e.g. Antonov *et al.*, 1988). However, there are several limitations which are discussed at length by Werman *et al.* (1996): **1)** The analysis is restricted to single-copy genes. **2)** High intraspecific

polymorphism can be problematic to estimate phylogenetic relationships of closely allied species. **3)** DNA-DNA hybridization is not only technically complex, it is also relatively expensive compared to other molecular techniques. **4)** Radioisotopes are used. **5)** In many cases considerable amount of DNA is needed, limiting the number of taxa that can be studied. **6)** Analysis of data requires that reciprocal reactions are carried out, i.e. reaction of species A (as tracer - labelled DNA) to B (as driver - unlabelled DNA) and vice-versa; so, if many taxa are being examined the number of reactions needed will be very large. **7)** There is the possibility that we are measuring genetic distances between paralogous sequences [only orthologous genes are homologous, paralogous genes have evolved independently after an ancestral gene duplication event – see also Doyle, 1992, p. 148-150]. **8)** Different amounts of DNA per genome (often the case between different species) will bias the measurements in the hybridization reaction.

Despite all the limitations discussed by Werman *et al.* (1996), these authors still considered that DNA-DNA hybridization is a "practical technique [...] to assess evolutionary relationships". However, the use of this method in phylogenetic studies is certainly in decline, and I tend to agree with Bachmann (1992) that "DNA-DNA hybridization is now only of historical interest in [plant] taxonomy".

2) RFLPs

Restriction Fragment Length Polymorphism (RFLP) relies on the use of **restriction endonucleases** (or REs), which are enzymes isolated from bacteria that digest (break) DNA at very specific sites [e.g. the enzyme *Hin*dIII, obtained from *Haemophilus influenzae*, cleaves DNA every time it encounters the following sequence: 5'–A↓AGCTT–3'; another enzyme, *Eco*RI, extracted from *Escherichia coli*, cleaves DNA at: 5'–G↓AATTC–3']. Thousand of these enzymes have been already isolated. RFLPs methods are sometimes referred as **restriction site analysis** or, simply, 'restriction analysis' (Dowling *et al.*, 1996).

Nucleotide substitutions change the DNA sequence and consequently the position of cleavage sites. Also, insertions and deletions (**indels**) will affect the length of fragments produced by a particular restriction enzyme, and may also introduce or eliminate cleavage sites. As a result, the number and size of fragments

originated by the same enzyme may differ between different taxa, and this polymorphism can be used as character data to infer phylogenetic relationships. For further explanation see Brettschneider (1998), and Dowling *et al.* (1996).

RFLPs techniques are summarised in Appendix III(2).

It is generally agreed that, ideally, **restriction site maps** (or cleavage maps) should be constructed before analysis of data (i.e. the restriction sites for each enzyme are located along the sequence examined – either the DNA probe or the gene that was amplified by PCR). One mapping method involves the use of *single* and *double digests* as described below (for detailed explanation of this and other mapping methods, see Dowling *et al.*, 1996, p. 302-314):

1) Single digests (i.e. reactions using single restriction enzymes) and double digests or 'pairwise' reactions (two enzymes at a time) are carried out for all the enzymes and in relation to each other.

2) The fragment patterns obtained by single and double digests are compared, this allows to infer the position of restriction sites. It is recommended to start with the enzymes that produce the lowest number of fragments, i.e. that have the smallest number of restriction sites, and then, progressively, add the data from other enzymes until all the restriction sites have been mapped.

If mapping is done characters states will be presence or absence of restriction sites. However, mapping restriction sites is very time-consuming, and some authors have chosen to use presence or absence of bands (restriction fragments) as the character states. Dowling *et al.* (1996) argued that the use of fragments as characters "should be restricted to very closely related sequences [species], and then used with caution"; but they encouraged the use of mapping as "well worth the investment". Swofford *et al.* (1996, p. 412-413) strongly advocates for mapping restriction sites, as they considered that fragment data alone is not reliable in phylogenetic studies.

According to Jansen *et al.* (1998), the main advantages of RFLPs are that: **1)** If several restriction enzymes are used, it is possible to sample more sequence diversity and more informative sites, than sequencing single genes (e.g. in chloroplast DNA, restriction site analysis provides as much information as sequencing 2-3 of its genes). **2)** A large portion of the genome can be surveyed and, therefore, the analysis is more likely to provide a correct phylogeny than using single or contiguous genes.

The main limitations of RFLPs can be summarized as follows: **1)** The method requires intensive work and is expensive, especially if mapping is carried out. **2)** A large amount of highly purified DNA is required, thereby, large samples of plant material, either fresh or preserved in silica-gel, need to be available [except in the PCR-RFLP method, but in this case, only a very small part of the genome (often a single gene) is examined]. **3)** Restriction site data from different laboratories is not easily combined, due to differences in preparation of gels, which affects the estimation of fragment sizes and positioning of restriction sites. **4)** Loss of a restriction site may have originated by different mutations (a recognition site normally involves 4-6 bp), but it will be score as a single event; this is specially relevant if the sequence has a high rate of mutation. **5)** Radioactive isotopes have often been used (but, there are now non-radioactive choices). **6)** The availability of cloned DNA-probes (for Southern blotting) may restrict what can be surveyed in the genome – Bachmann (1992), Brettschneider (1998), and Jansen *et al.* (1998).

The use of RFLPs has decreased in the last few years, but the technique is still an important source of data at various levels of the classification, from the study of genera and closely related species, to higher taxonomic levels, especially in chloroplast DNA (cpDNA) – Jansen *et al.* (1998); Soltis & Soltis (1998). Restriction site analysis of cpDNA have also been carried out in the *Umbelliferae* (Plunkett & Downie, 1999). These authors confirmed that restriction site analysis provides many more (2.6-3.6 times in this study) potentially informative characters than sequencing of single genes.

3) RAPDs

Randomly Amplified Polymorphic DNAs (RAPDs) is a relatively simple technique based on the use of **PCR** (see section 9.2.5), but in which the *primers* (oligonucleotides) are 'arbitrarily' chosen [primers are the 'starting point' for DNA duplication]. PCR is normally used to amplify a specific segment of DNA, using two different primers that bind opposite strands in the flanking regions of this segment (part of the sequence of the flanking regions needs to be known to design specific primers). In the case of RAPDs, the primers used have a known sequence, normally of 10 base pairs (bp), that is chosen at random; so, it is possible to use an enormous

number of different primers. During PCR, the RAPD primers will find and anneal (hybridize) to many complementary sites throughout the genome. However, not all these hybridizations will lead to the production of [visible] PCR products (i.e. bands of DNA in the gels). To obtain a PCR product, it is necessary that two primers (the same or different) will bind in opposite strands within a distance of 2 kb (2000 bp) from each other; only then the exponential amplification of the segment can occur (Edwards, 1998).

The technique can be summarised as follows: **1)** Total DNA is extracted from plant tissue. **2)** PCR is carried out using several primers with random sequence. **3)** PCR products (amplified DNA fragments) are run in a gel (electrophoresis), which is later stained with ethidium bromide for visualisation under UV light.

Although the technique have become fairly popular in recent years, mostly for its simplicity and speed, it has very serious limitations which are discussed below:

PCR amplification with RAPDs is extremely sensitive to change of conditions, so everything needs to be precisely defined, otherwise there will be variation in the band pattern obtained for a single sample. As a consequence, concentration of all PCR components need to be accurately determined (e.g. quantity and quality of DNA and primers, etc), the conditions of PCR need to be standardised (i.e. type of PCR machine used, program of amplification chosen; even position of tubes in the PCR machine may affect amplification), the way the samples are prepared, etc. (Edwards, 1998).

Jones *et al.* (1998a) presented the results of "reproducibility testing of RAPDs" in 9 European laboratories. Despite all the care in using exactly the same package of components (DNA samples, primers, DNA polymerase, buffer and agarose to prepare the gels) and efforts to maintain constant conditions of PCR, the results of this testing showed a considerable lack of reproducibility: only 2 out of the 9 laboratories produced the same band pattern ('profile') for the same DNA sample. Edwards (1998) suggested that, despite loss of data, this problem could be reduced if only bands with an intensity above a certain threshold were counted. But, even if this is done, the mentioned profiles of the tests of reproducibility (see Fig. 9.4 in Jones *et al.*, 1998a) will still be different for a single sample, as 2 of the profiles had "strong" bands that were not present in the others.

Jones *et al.* (1998a) concluded that RAPDs may be used to study biodiversity within a single laboratory, but not if results need to be exchanged between institutions. But they added, that even within a single laboratory the RAPD profiles may be proven difficult to reproduce if there are changes in equipment, goods or "even personnel".

RAPDs may have become quite popular, but at present the results are living up to the name of the technique: they are also a little at "random" and, therefore, not reliable.

4) AFLPs

Amplified Fragment 'Length' Polymorphism (AFLP) is a recently developed technique (Vos *et al.*, 1995) that combines the use of **restriction endonucleases** and the **PCR**. The name AFLP was chosen because of the resemblance with the RFLP technique (see above), the main difference being that AFLP uses PCR to detect a subset of the restriction fragments, while RFLP uses Southern hybridization. However, the authors (Vos *et al.*, 1995) remarked that the name AFLP should not be used as an acronym, because the technique do not show length differences, but rather presence or absence of fragments. As in RAPDs (see above) no previous knowledge of DNA sequence is necessary, and the primers are arbitrarily chosen. The technique is summarised in Appendix III(3).

AFLPs have several advantages: **1)** It is a fast technique that is less labour intensive than RFLPs. **2)** It can produce a large amount of information. **3)** High reproducibility of results which are little or not affected by variation of PCR conditions such as concentration of DNA (Matthes *et al.*, 1998; Wolfe & Liston, 1998). As described by its authors (Vos *et al.*, 1995), AFLP combines the "reliability" of RFLPs "with the power of the PCR". Indeed, AFLP has been confirmed a reliable technique by Jones *et al.* (1998b). These authors presented the results of reproducibility testing in 7 European laboratories and, for a single sample, the same pattern of bands was obtained by all. The only exception was a single missing band in one of the gel profiles that could apparently be explained by different DNA preparation in that laboratory.

Nevertheless, the AFLP technique has some limitations: **1)** Its use is restricted to population studies and distinction or identification of individual genomes. **2)** Radioactive isotopes have been used – there is now the choice of using fluorescent labels, but then an automated sequencer (an expensive machine) is required (Wolfe & Liston, 1998). **3)** Although AFLPs are far more reliable than, for instance, RAPDs, quality of DNA and PCR machine used may still affect the results (band patterning), and so it is recommended to assess reproducibility by carrying out two reactions in parallel for a single sample (Matthes *et al.*, 1998). Wolfe & Liston (1998) considered another limitations: the technique now includes two steps of amplification (PCR) [a modification introduced to the original protocol (see Mathes *et al.*, 1998)] and it is necessary to use polyacrylamide gels, while other PCR-based methods only require agarose gels stained with ethidium bromide. Nevertheless, Wolfe & Liston (1998) remarked that the few limitations of AFLPs are largely compensated by its advantages.

5) Microsatellites and minisatellites (STRs, SSRs, VNTRs)

The genome of eukaryotic cells consists of two types of sequences: **single copy DNA** (unique genes) and **repetitive DNA**; the latter is formed by basically identical copies of sequences with a few to thousands of base pairs each. The number of copies of a repeat unit goes from only a few to millions, which are either found dispersed through the genome or arranged in tandem arrays, in the same or in different chromosomes. A part of this repetitive DNA are genes that code for products that are necessary to the cell in large amounts; a good example is the nuclear ribosomal DNA (nrDNA) – the ITS region is part of the repeat unit of the nrDNA (see DNA sequencing below, and Fig. 9.1). However, most of the repetitive DNA has no known function, containing mainly noncoding and nontranscribed sequences. Highly repeated DNA is also called '**satellite DNA**', because when genomic DNA is fragmented and separated by ultracentrifugation in a density gradient, the highly repeated sequences form distinct bands ('satellites') separated from the main band of DNA (Griffiths *et al.*, 1996, p. 504-513; Li, 1997, p. 384-388).

Jeffreys *et al.* (1985) discovered that there is high variation in length in certain dispersed tandem repeats in the human genome, and that the differences in length were due to variation in the number of repeat units. They designated these small repeat units (10-15 bp long) as '**minisatellites**'. These authors applied the principle of RFLPs (see above) to detect the different sized tandem repeats. Basically, they digested genomic DNA with restriction enzymes that do not cut within the repeat unit; the restriction fragments were separated by electrophoresis and hybridized by Southern blotting with a radioactively labelled probe of a sequence that was known to be present in repeats. The results showed high variation in the bar code-like band pattern, which was found to be specific to single individuals, and could, therefore, be used as a **'DNA fingerprint'.** The bands in a single fingerprint represent alleles (tandem repeats) of different loci, with each locus generally having multiple alleles of different lengths that show Mendelian inheritance; each fingerprint represents then a 'multilocus profile' (Bruford & Saccheri, 1998). The use of DNA fingerprinting had a tremendous impact in the study of genetics of populations and, more notoriously, in forensic medicine and parentage testing.

Minisatellites, with a repeat unit 10-200 bp long, and other tandem repeats with smaller units (2-6 bp long), the '**microsatellites**', are found in all eukaryotic genomes [the length values given 'by definition' to minisatellites and microsatellites differ among authors]. Microsatellites are also known as *Short Tandem Repeats* (**STRs**), or *Simple Sequence Repeats* (**SSRs**). Both, minisatellites and microsatellites are known as *Variable Number of Tandem Repeats* (**VNTRs**) – Brufford *et al.*, 1998; Ciofi *et al.*, 1998. The high variability in length of VNTRs is explained by the occurrence of '*slippage*' during DNA replication (see e.g. Li, 1997, p. 27-29, 391-392).

In recent years, VNTRs have also been studied using a PCR-based technique, which also allows the use of herbarium material or rare plants as much smaller amounts of plant material are required (Crawford, 1997). In this case, primers are designed to be complementary to the flanking sequences of VNTRs loci. However, this technique has been more often applied to microsatellites (STRs or SSRs), because many minisatellites alleles (tandem repeats) are often too long for PCR amplification – total length of minisatellites can be as much as 50 kb (50,000 bp);

PCR is normally not possible in fragments much longer than 10 kb (microsatellites repeats are generally shorter than 500 bp). Also, primers developed for microsatellites of one species, can be used in related species, which generally is not possible in the case of minisatellites (Bruford *et al.*, 1998; Ciofi *et al.*, 1998). Another advantage of the use of PCR to study microsatellites is that it allows the study of allelic distribution in single locus (Jones *et al.*, 1998c). For further details on the techniques involving minisatellites and microsatellites see: Bruford *et al.* (1998), Bruford & Saccheri (1998), Ciofi *et al.* (1998), and Dowling *et al.* (1996).

VNTRs, in particular microsatellites, have been very valuable in population genetics (e.g. linkage mapping), and ecological and conservation studies. Allozymes (see protein studies above) provide similar data (i.e. codominant allelic variation), but polymorphism of microsatellites is almost always much higher (Bachman, 1997; Ciofi *et al.*, 1998). An important advantage of the study of microsatellites is their reproducibility, which was tested in 9 European laboratories, with alleles being "faithfully reproduced" for each sample (see Jones *et al.*, 1998c).

The major limitation of VNTRs is that their use in systematics is restricted to population level or lower.

Note: The term 'DNA fingerprint' was initially used only for the band patterns obtained from the study of minisatellites, and later microsatellites, but at present is also used for other techniques that provide multilocus profiles, such as RAPDs and AFLPs analysis (see above) – Bruford & Saccheri (1998).

6) DNA sequencing

DNA is the genetic material and ultimately the best source of data for phylogenetic studies. However, it was only after the development of the polymerase chain reaction (PCR), and more recently with the use of automated sequencers, that DNA sequencing became a routinely used technique in molecular systematics. Until a decade ago, the use of DNA sequences in plant phylogeny had been mostly restricted to one chloroplast gene, *rbc*L, which encodes the large subunit of *rubisco* (ribulose-1,5-bisphosphate carboxylase), the enzyme that catalyses carbon fixation in the photosynthesis (Palmer *et al.*, 1988; Olmstead & Palmer, 1994). Recent years have seen an extraordinary increase in DNA sequencing, so that now the amount of sequence data accummulated is astonishing. Several genes (mainly chloroplast, but

also nuclear genes) have been widely sequenced in plants, and used in phylogenetic analysis (Soltis & Soltis, 1998; see also Judd *et al.*, 1999).

Sequencing methods (including protocols), their applications and limitations, are comprehensively discussed by Hillis *et al.* (1996c). See also section 9.2 for detailed description of the sequencing method used in the present study.

The main advantages of sequence data are: **1)** Nucleotides are the basic units of information in organisms. **2)** The amount of data that can be potentially surveyed is immense. **3)** As different genes show distinct rates of mutation, there are genes suitable to study relationships at all ranks in a classification. **4)** Sequence data are essentially character data and can be easily used in phylogenetic analysis – Hillis *et al.* (1996c); Swofford *et al.* (1996).

The main limitations of sequencing are: **1)** The technique is still expensive. **2)** Sequencing in some groups of plants can be technically very troublesome. **3)** Sometimes is difficult to distinguish orthologous from paralogous sequences (see 'nuclear DNA', below). **4)** Selecting genes suitable to investigate relationships in a particular taxonomic group is not always straightforward (experience with other plant groups can be used, but groups of the same rank are not necessarily equivalent in the genetic divergence between their members). **5)** Ambiguities plus errors in alignment of sequences (i.e. failure in establishing homology of nucleotide position) will lead to erroneous phylogenetic interpretation.

In plants, genes for sequencing may come from 3 different genomes: nuclear, chloroplast and mitochondrial. The following is a brief discussion of the different characteristics of these genomes and their current use in molecular systematics. See Soltis & Soltis (1998) for a review of the genes or DNA regions that have been more frequently used in phylogenetic analysis.

Chloroplast DNA

Comparative DNA sequence analysis initially concentrated in the chloroplast DNA (cpDNA), mainly because it has a small size (typically 135-160 kb), simple uniform structure (circular molecule with essentially the same gene arrangement in most land plants), and, as it contains mostly single-copy genes, sequence homology can be easily established. Also, cpDNA is typically inherited from a single parent, as a unit not subjected to recombination. Therefore, its evolution is comparable to that

of clonal lines, which simplifies phylogenetic reconstruction. Chloroplast DNA is essentially a conservative molecule, and its genes, displaying various rates of mutation, have been informative in the differentiation of genera and upper levels of the classification. However, cpDNA has had very limited used at infrageneric levels. There is another important disadvantage in using cpDNA: as this molecule is cytoplasmically inherited, it may pass from one species to another by 'introgression' after an hybridization event, or by random inheritance and extinction during 'lineage sorting'. Both process will lead to erroneous phylogenetic interpretation – Bachmann (1992 & 1997), Downie *et al.* (1996); Olmstead & Palmer (1994), Palmer *et al.* (1988), Plunkett *et al.* (1996a,b & 1997), Plunkett & Downie (1999); Soltis & Soltis (1998). For further explanation on introgression and lineage sorting see Doyle (1992).

Mitochondrial DNA

Angiosperm mitochondrial DNA (mtDNA) varies enormously in size, structure and gene order. Although largely used in the study of animals, mtDNA has been poorly studied in plants and little used in phylogenetic analysis. Mitochondrial DNA has a much slower rate of mutation than cpDNA, and so it could potentially be used to investigate phylogenetic relationships at the highest levels of the classification (Olmstead & Palmer, 1994). However, the rapid changes in structure, size and gene arrangement of plant mtDNAs, have made these molecules very difficult to analyse (Soltis & Soltis, 1998).

Nuclear DNA

The nuclear genome is immense and very complex in structure, however in recent years its study has significantly increased. Interest in sequencing nuclear genes stems from problems found when using the chloroplast genome (see above) and the need of additional 'markers' in phylogenetic analyses, in particular at lower levels (genera and species) where fast evolving genes are needed (Bachmann, 1992 & 1997; Baldwin, 1992).

One of the major difficulties in studying nuclear genes is homology evaluation, i.e. how to distinguish between **orthologous** and **paralogous genes**. Orthologous gene sequences have derived from speciation (i.e. the same gene diverged between species), while paralogous sequences are the result of independent evolution after the duplication of an ancestral gene (see e.g. Doyle, 1992, p. 148-150;

or Moritz & Hillis, 1996, p. 7-9). Only orthologous sequences (= orthologues) are homologous in a phylogenetic context, where **homology** is a synonym of **synapomorphy** – [For extensive arguing on this matter and the different concepts of homology ("classical, evolutionary, phenetic, cladistic, and utilitarian"), see Patterson (1982); for further discussion on homology in molecular characters, see Doyle & Davis (1998)].

Many nuclear genes have multiple copies at different loci or are part of multigene families, which contain genes that have derived from the replication of an original single gene. So, there is a high risk that we may 'mix' in our analysis orthologous and paralogous sequences, leading to incorrect tree topologies.

However, some of these multigene families, and basically all classes of repetitive DNA, undergo a process generally designated as **concerted evolution** (also known as *horizontal* or *coincidental evolution* – Li, 1997, p. 309), where the multiple copies of the gene (repeat units) maintain a very high homogeneity within a single individual and species, while tending to diverge among species. This phenomenon has been explained by the still inadequately known mechanisms of '**molecular drive**', which include *gene conversion*, *unequal crossing-over*, *replicative transposition* and *slippage* (Dover, 1982; Elder & Turner, 1995; and for detailed discussion on these mechanisms see e.g. Li, 1997).

There are several advantages on sequencing repetitive genes homogenized by concerted evolution: **1)** These genes are easily detected because of their very high numbers of copies in the genome. **2)** As the many different copies of the gene tend to become identical in single organisms, and consequently in the population and eventually in the species, the problem of paralogues is basically eliminated, because the multiple copies of the gene evolve as if they were single units.

However, there could be serious limitations when using repetitive sequences:

1) If concerted evolution does not efficiently homogenize all copies of the gene under study, we will find different repeat variants in a single organism. The first problem will be obtaining the sequences (especially if there are insertions and/or deletions), as they will appear 'superimposed' to each other and will result in many unidentified nucleotides. In this situation, the only way to retrieving clear sequences is by cloning. However, the most serious problem resulting from 'failure' of concerted evolution is the possibility of sampling sequences with different

evolutionary histories (Baldwin *et al.*, 1995) – i.e. we have again the problems of paralogues.

2) Efficient concerted evolution will hide hybridization events (reticulation of the phylogenetic tree) as, in theory, only one of the repeated genes coming from the two species will be preserved in further generations.

Nuclear ribosomal DNA and ITS region

The best known example of a repetitive gene region under concerted evolution is the nuclear ribosomal DNA (nrDNA). Fig. 9.1 shows a diagram of nrDNA gene arrangement.

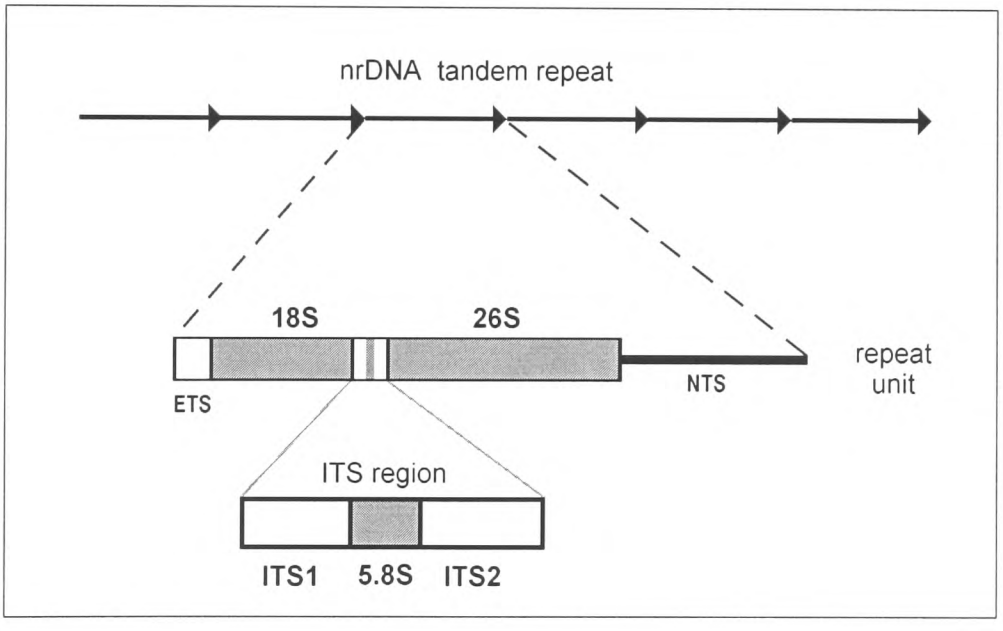


Fig. 9.1 - The nuclear ribosomal DNA (nrDNA) is arranged in tandem repeats, located in the nucleolar organizing region. Each repeat unit contains a nontranscribed region (**NTS** or nontranscribed spacer), and a transcribed region (represented in blocks). The latter includes three **coding regions** (shaded areas) of ribosomal RNAs (**18S**, **5.8S**, **26S**) and the transcribed spacers: **ETS** (external transcribed spacer), and **ITS 1** and **ITS 2** (internal transcribed spacers). The **ITS region** includes ITS 1 and ITS 2 and the coding region of the 5.8S. The ETS and NTS together form what has been designated **IGS** (intergenic spacer) – diagram and information based on Schaal & Learn (1988); Jorgensen & Cluster (1988); Bachmann (1992); and Soltis & Soltis (1998).

In the particular case of **nrDNA** (Fig. 9.1), we have regions with very different rates of mutation that are potentially useful at various levels of the classification:

The coding regions of **18S** and **26S** (25S or 28S in other eukaryotes) are very conservative and have been successfully used at familial rank and above; the **5.8S**, another coding region, is too short to provide sufficient informative variation – [the 18S nrDNA encodes for the 18S rRNA of the small subunit of ribosomes, while 26S and 5.8S encode for the respectives rRNAs of the large subunit] – Hillis & Dixon (1991); Soltis & Soltis (1998).

The *intergenic spacer*, **IGS** (see Fig. 9.1), is a fast evolving region, but its complexity (variable subrepeat structure) and considerable length variation (even within species and populations) have made it a very difficult region to analyse – Hillis & Dixon (1991); Soltis & Soltis (1998).

The rapid evolving regions of the *internal transcribed spacers* (**ITS1** & **ITS2**) have been useful and largely used to investigate phylogenetic relationships among species and allied genera (e.g. in the *Umbelliferae* – Downie & Katz-Downie, 1996; Downie *et al.*, 1998; Valiejo-Roman *et al.*, 1998; Katz-Downie *et al.*, 1999). The function of the ITS spacers is not clearly known, but the ITS transcripts, which are not incorporated into ribosomes, seem to be involved in the maturation of nrRNAs. So, despite high variability, ITS is under some evolutionary constraints, being fairly conservative in length in flowering plants, which facilitates sequence alignment – Baldwin (1992); Baldwin *et al.* (1995); Hillis & Dixon (1991); Soltis & Soltis (1998).

9.1.2 Methods of data analysis

It is far beyond the aims of this project to discuss the multitude of data analysis approaches available for molecular systematists. For an introduction and comprehensive discussion on methods of phylogenetic analysis see e.g. Li (1997), Page & Holmes (1998) and Swofford *et al.* (1996). A few aspects of data analysis are discussed below; others are referred to in sections 9.2.8 & 9.2.9, or when explanation is needed.

The different techniques discussed in the previous section provide data that can be classified either as distance data or discrete data.

A) Distance data

Distances (coefficients of similarity or dissimilarity) in a data set are numerical values calculated as pairwise comparisons (two taxa at a time, and between all), which are assumed to be a measure of genetic divergence. All data obtained in molecular systematics can be analysed as distance data. Some techniques provide data that is only suitable for distance analysis, such as: protein immunology (serology), DNA-DNA hybridization and RAPDs. Discrete data can be converted into distances and analysed by distance methods, but part of the information is lost in the transformation of the data (see e.g. Page & Holmes, 1998, p. 172-173, 186). DNA sequences are basically discrete data, but can be transformed into genetic distances: two sequences are compared at a time, and a distance value reflecting the number of nucleotide differences is calculated. Distance data are not suitable for cladistic analysis as they cannot be expressed as character (state) variation – distance data cannot be converted into discrete data.

Examples of methods of distance analysis are: the clustering method **UPGMA** (*Unweighted Pair Group Method with Arithmetic Means*), and the **neighbour joining** and **minimum evolution** methods (see Li, 1997, p. 105-112; and Page & Holmes, 1998, p. 179-187). Methods that use distance data have been often termed 'phenetic' (see below) as they have been widely used in the numerical analysis of phenotypic characters (mainly morphological).

B) Discrete data

Discrete data are basically *character data*. Discrete characters, by definition, have clearly defined (character) states, which can be represented as finite numerical values, generally integers. In contrast, continuous quantitative data/characters can have, virtually, all possible values, e.g. size, length, etc. Discrete characters can be binary (two character states, represented as 0/1 – e.g. presence/ absence of a particular restriction site) or multistate (3 to many character states – e.g. nucleotides: 4 states). Cladistic analysis requires discrete data, i.e. characters that have clearly

defined states that can be ordered in a hypothetical transformation series from a plesiomorphic (ancestral) state to an apomorphic (derived) state. Character transformation series are used to establish relationships between taxa in the form of a phylogenetic tree or cladogram.

The most important methods that use discrete data are **maximum parsimony** and **maximum likelihood**. Maximum parsimony is the most widely used cladistic method; here the optimal phylogenetic tree is chosen for showing the fewest evolutionary changes, because the shortest tree explains the data in the most simple and efficient way. Maximum likelihood chooses the tree with the highest probability of explaining the present data, i.e. it favours the tree that makes the observed data the most likely evolutionary outcome. For detailed explanation on these methods see Lewis (1998), Li (1997, p. 112 –119), Page & Holmes (1998, p. 187-201), and Swofford *et al.* (1996, p. 415-446).

Of the techniques discussed above (section 9.1.1), only sequencing of DNA (or RNA) or proteins, and restriction site analysis (RFLPs) produce discrete data which can be unambiguously used in phylogenetic (cladistic) analysis. Isozymes (allozymes), AFLPs and VNTRs (microsatellites and minisatellites) studies produce discrete data: presence/absence of bands (i.e. alleles). However, character independence, an assumption of cladistic analysis, is often violated when using alleles as "characters", as the "states" (alleles) may be present at the same time in single organisms (heterozygotes) – Swofford *et al.* (1996). In these cases it is more appropriate to use the methods of analysis of population genetics (see e.g. Weir, 1996).

C) Cladistic versus phenetic approaches

Cladistics and phenetics are different approaches to the numerical analysis of relationships and classification of organisms. **Phenetics** establishes classifications based on overall similarity, and traditionally uses large numbers of phenotypic characters (hence the designation 'phenetic'), although nowadays genetic data are also used. For description of the principles of phenetics see: Sokal & Sneath (1963), Sneath & Sokal (1973), and Pankhurst (1991). **Cladistics** attempts to determine the phylogeny of organisms, defining monophyletic groups that are then the base of

classifications. For detailed discussion on cladistics see: Wiley (1981), Wiley *et al.* (1991), Forey *et al.* (1992), and Kitching *et al.* (1998).

After more than 30 years since the publication in English of Hennig's ideas on '*Phylogenetic Systematics*' (Hennig, 1965 & 1966), which gave raise to what is known as cladistics, the dispute between 'cladists' and 'pheneticists' is still not over. Patterson (1980) compared "the virulence of this controversy [...] in so old and apparently harmless a discipline as systematics" with the violent arguments generated by Darwin's theory of evolution.

The main criticisms to phenetics are: **1)** The occurrence of parallelism and convergent evolution is ignored by simply using similarity; this can lead to the definition of artificial groups (Wiley *et al.*, 1991). **2)** Although phenetic methods do not follow any phylogenetic assumptions, the tree-like phenograms (dendrograms) have been sometimes wrongly interpreted as if they reflected evolutionary history (Judd *et al.*, 1999, p. 30-31).

The main criticisms to cladistics are: **1)** Several subjective assumptions are made, such as the definition of ingroups and outgroups, and polarization of characters (which character states are plesiomorphic and which are apomorphic). **2)** A branching tree (cladogram) without reticulation (i.e. with no hybridization events) is known not to be always an accurate representation of how evolution took place. **3)** Parsimony is a very simplified hypothesis of evolution; the shortest tree is not necessarily the true phylogeny; also, when many taxa are included, it is not possible to guarantee that the shortest tree will ever be found (computationally impossible to examine all possible trees for more than 20 taxa – or even less depending on size of data matrix). There are other criticisms to cladistics (for further details see Pankhurst, 1991, p. 86-87; and Pankhurst, 1995), but most of them had significant validity at a time when mainly morphological data was being used. However, the panorama has changed with the advent of sophisticated molecular studies, in particular DNA sequencing. If a putative ingroup is not really monophyletic, analysis of several molecular markers should clearly show so.

Occurrence of hybridization is difficult to estimate, and it will always represent a problem in phylogenetic interpretation. However, the use of different genes, in particular nuclear genes, can help to detect if gene introgression have

occurred as conflicting results will be obtained. More recently, other phylogenetic methods have been developed which produce '*networks*' (graphs including reticulation) instead of trees, and therefore can more accurate represent the relationships in groups where hybridization is suspected or is known to commonly occur – see Hillis *et al.* (1996b) and Swofford *et al.* (1996).

Considering molecular data (in particular sequences), pheneticists have argued that because we will never know the true phylogeny of life, even if we sequence all sort of genes, we may better use all information available, and not only what under the assumptions of parsimony are 'informative sites' (see e.g. Li, 1997, p.112-115). But not even phenetic approaches use all the data available, as part of the information is lost in the transformation into distances. Nevertheless, distance methods of analysis are still important when testing competing hypothesis, and should not be dismissed by mere principle; in complete sets of data distance and discrete methods produce very similar or even identical tree topologies (see e.g. Page & Holmes, 1998, p. 173).

We will never know what exactly happened during evolution, but the aim of systematics has long been to classify into natural groups, and I believe that 'natural' generally means monophyletic; the exception being the groups that resulted from hybridization of taxa with different evolutionary histories. Nevertheless, natural hybrids generally occur between closely related species, and so monophyly, in a broader sense, is only rarely 'broken'. Now that genetic data are finally becoming widely available, we have a chance of getting an approximation to this ideal 'natural' classification, at least with the organisms that are still living on the planet – classification of extinct organisms is, of course, another matter.

9.1.3 Choice of methods

Several of the references indicated in the previous sections of this chapter have been published very recently, and, obviously, had no influence in the choice of method for this project. However, **DNA sequencing** has long been regarded as the ideal source of data for phylogenetic investigation, and, in the last few years, it finally became a routinely used technique in molecular systematics.

The **ITS region** of nuclear ribosomal DNA, including the fast evolving spacers ITS1 & ITS2, was chosen for initial sequence analysis in *Bupleurum*, as it had been successfully used in phylogenetic research at the generic and infrageneric levels in several plant groups (Baldwin, 1992; Baldwin *et al.*, 1995), including the *Umbelliferae* (Downie & Katz-Downie, 1996). For full explanation of the characteristics and advantages of this gene region, see section 9.1.1B (DNA sequencing).

As method of phylogenetic analysis, **maximum parsimony** was chosen because it has been thoroughly investigated and widely used, also the concept of parsimony is fairly easily understood, i.e. simple hypotheses are favoured to more complex ones [maximum likelihood has only recently started to be more frequently used, and its theoretical principles are rather more difficult to understand].

9.2 Material & methods

9.2.1 Preparation of plant material for molecular analysis

The earlier systematic studies that included DNA analyses mainly used material from plants easily available in cultivation or with large temperate distribution and hence readily accessible. However, soon it became necessary to expand the source of plant material as many rare species were not available in botanical gardens. As a consequence, it became necessary to obtain good samples for DNA studies during field work. Fast freezing (cryopreservation), using liquid nitrogen or by placing material in freezers at very low temperatures (c. -80°C), is the best method to preserve DNA in plant tissue. But freezing facilities are generally not available during field work. Traditional methods of preservation such as chemical treatments (formaldehyde, ethanol, chloroform, etc) cause considerable DNA degradation, but, in contrast, simply drying proved an effective method to preserve DNA integrity (Doyle & Dickson, 1987). Chase & Hills (1991) demonstrated that silica gel is an excellent material for rapid desiccation and preservation of fresh material collected in the field.

Herbarium material is another possible source for molecular studies. In general, DNA obtained from herbarium specimens shows some degree of degradation, the extent of which much depends on how old is the specimen, how fleshy was the plant, its particular chemical composition, and how long it took to dry – long drying causes metabolic changes in the tissues that will affect the quality of DNA and cause difficulties during its extraction and/or amplification (Savolainen *et al.*, 1995). Nevertheless, herbarium material of some groups of plants has been successfully used in molecular studies, including sequencing – e.g. in *Umbelliferae* (Plunkett *et al.*, 1996b & 1997).

In the present work, fresh material was preferentially used if available. However, herbarium material was used for most of the species/taxa as fresh material could not be obtained (42 samples were taken from herbarium specimens out of a total of 68 sequenced). The following is the protocol used for **fresh material** (adapted from Chase & Hills, 1991):

1) Prepare several small polythene bags (if available ziplock bags are more practical) with 30-100 g of silica gel (28-200 mesh size – Sigma S-4883), including 5 % or more of the self-indicating type (silica gel Type III, Sigma S-7625).

2) Collect fresh leaves (preferably, but other material such as herbaceous stems, inflorescences or 'dry' fruits can also be used) and place them in the silica gel bags. It has been recommended that young leaves should preferably be used as they often contain smaller amounts of polysaccharides, but in general undamaged fully mature leaves are adequate (Hyam, 1998). If leaves are large (>5 cm long/wide) they should be torn in smaller pieces. Normally, the amount of plant material is 1 to 10 of silica gel, but if material is fleshy or is slightly damp because of the weather conditions, more silica gel should be used (if material is wet, dry the surface first). Distribute (shake) silica gel evenly between the leaves.

3) After 24 hours check the colour of the indicating silica gel (dark blue if dry, pink if humid) and if the leaves are dry ('crispy'). If leaves are not dry, and silica gel seem saturated (crystals of the indicating type are pink), it should be replaced with dry silica – in the case of *Bupleurum* this was never necessary, but this might be applicable for more succulent material.

4) After 2-3 days, remove the used silica gel (recommended, but not essential if silica is not saturated) and place in the bags a small amount of dry indicating type of silica gel; this will allow to check that material is kept dry. The used (humid) silica gel can be re-used by drying in an oven at 150°C or 175°C for 30 minutes up to 1 hour. But, to avoid contamination, it is essential to check first that no leaves or other plant material are left in the silica – often minute pieces of leaves can be seen and, although it might seem tedious, they must be removed (use a pair of tweezers). Do this before drying the silica as the pieces of leaves turn brown with the heat and will be difficult to see afterwards.

5) Dried samples can be store at room temperature for several months without significant deterioration of DNA. However, for longer periods (often the case) is recommended to store the samples in a freezer, at -20°C, but preferably at -80°C.

Material from **herbarium specimens** is already dry, but it is necessary to confirm that there is no humidity left (e.g. absorbed from the atmosphere). Therefore,

prior to use, the material should be placed in small plastic bags containing a small amount of silica gel (including indicating type), and left overnight.

Table 9.1 summarises the information of the 68 sequenced plant accessions. Complete information on accession numbers is given in Appendix II. All material used was collected or taken by myself from living plants (wild or cultivated) or herbarium specimens, with the exception of one sample: material of *B. salicifolium* was kindly collected by Martin Gardner and Sabina Knees (RBGE, Royal Botanic Garden Edinburgh) in the Canary Islands. Eight samples were collected from cultivated material, 6 from plants cultivated by myself and two (*B. angulosum* and *B. mundii*) from plants that were already growing at RBGE. The two herbarium specimens used of *Anginon* Raf. were identified following Allison & Van Wyk (1997).

Table 9.1 - Plant accessions of *Bupleurum* and *Anginon* sequenced for ITS.

F = fresh material; **H** = herbarium material; **W** = wild material; **C** = cultivated. Complete information on accession numbers is given in Appendix II. See Appendix I for explanation of herbaria abbreviations.

Taxon	Acc. No	Geographical area	Date of collection	F/ H W/ C	Voucher specimen
Anginon difforme	**193**	South Africa: Calvinia	21.viii.1990	H/W	All Batten 1018 (E)
A. paniculatum	**313**	South Africa: Central ridge	18.ii.1985	H/W	H.C. Taylor 11271 (E)
Bupleurum acutifolium	**228**	Portugal: Baixo Alentejo	16.ix.1996	F/W	S. Neves 24 (E)
	240	Portugal: Baixo Alentejo	08.viii.1997	F/W	S. Neves 27 (E)
	262	Spain: Málaga	05.ix.1997	F/W	S. Neves 64 (E)
	263	Spain: Málaga	05.ix.1997	F/W	S. Neves 65 (E)
	305	Spain: Málaga	17.v.1994	H/W	A.P. Latorre *et al.* (MGC 37708)
B. album	**269**	Morocco: Anti Atlas	10.vi.1974	H/W	Reading Univ./ BM Exped. 532 (RNG)
B. angulosum	**224**	Pyrenees	18.v.1998	F/C	S. Neves s.n. (E)
B. balansae	**268**	N Morocco: Oujda (area 19)	09.vi.1993	H/W	J. Molero *et al.* JMM-3198/5 (RNG)
	302	N Morocco: S Tetouan (area 2)	18.vi.1987	H/W	S.L. Jury *et al.* 8338 (RNG)
B. baldense	**276**	Spain: Guadalajara	21.vi.1995	H/W	M.A. Carrasco *et al.* (MA 558704)

Taxon	Acc. No	Geographical area	Date of collection	F/ H W/ C	Voucher specimen
Bupleurum barceloi	**295**	Balearic Islands: Mallorca	25.vii.1989	H/W	J. Orell Casasnovas (MA 474781)
B. benoistii	**285**	Morocco: S High Atlas	29.vii.1997	H/W	S.L. Jury *et al.* 18375 (E)
	300	Morocco: N High Atlas	21.vii.1966	H/W	R.M. & A.M. Harley 766 (BM)
	309	Morocco: S High Atlas	03.vii.1987	H/W	S.L. Jury *et al.* 8858 (RNG)
B. canescens	**301**	S Morocco: N Agadir	28.iii.1972	H/W	D. Bramwell *et al.* 265 (RNG)
B. canescens 'var. *handiense*' (= *B. handiense*)	**28**	Canary Islands	20.ix.1996	F/C	S. Neves s.n. (COI)
	207	Canary Islands: Lanzarote	15.v.1969	H/W	D. Bramwell 1631 (E)
B. dumosum	**293**	Morocco: NNE Asni	15.iii.1994	H/W	S.L. Jury *et al.* 14157 (RNG)
B. falcatum	**282**	Spain: Alava (Vi)	15.viii.1992	H/W	J.A. Alejandre 733/92 (MA 534085)
B. fruticescens ssp. ***fruticescens***	**238**	Spain: Huesca	30.ix.1987	H/W	G. Montserrat (MA 515853)
	253	Spain: Murcia	24.viii.1997	F/W	S. Neves 52 (E)
B. fruticescens ssp. ***spinosum***	**249**	Spain: Granada (Sierra Nevada)	16.viii.1997	F/W	S. Neves 42 (E)
	259	Spain: Cádiz	22.viii.1997	F/W	S. Neves 47 (E)
	280	Morocco: High Atlas	27.vii.1997	H/W	S.L. Jury *et al.* 18297 (E)
	311	N Morocco: W Rif (area 2)	23.vii.1995	H/W	M.A. Mateos *et al.* 6988/95 (RNG)
B. fruticosum	**243**	Portugal: Estremadura	10.viii.1997	F/W	S. Neves 33 (E)
	248	Spain: Málaga	15.viii.1997	F/W	S. Neves 41 (E)
B. gerardii	**17a**	Europe	03.viii.1994	F/C	S. Neves s.n. (E)
	67	Portugal: Beira Litoral	26.vi.1994	F/W	S. Neves 1 (E)
	306	Spain: Madrid	10.vii.1981	H/W	G. Navarro *et al.* (MA 310732)
	307	Spain: Granada	7.v.1980	H/W	J. Molero Mesa (MA 214590)
B. gibraltarium	**245**	Spain: Sevilla	15.viii.1997	F/W	S. Neves 35 (E)
	252	Spain: Murcia	24.viii.1997	F/W	S. Neves 51 (E)
B. lancifolium	**287**	N Morocco: Tanger (area 1)	21.iv.1995	H/W	S.L. Jury *et al.* 16552 (RNG)
B. lateriflorum	**279**	Morocco: S High Atlas	28.vii.1997	H/W	S.L. Jury *et al.* 18323 (E)
	303	Morocco: S High Atlas	02.x.1991	H/W	M. Ait Lafkih *et al.* 4939 (E)

Taxon	Acc. No	Geographical area	Date of collection	F/ H W/ C	Voucher specimen
Bupleurum longifolium	**310**	Germany: Baviera	17.vii.1993	H/W	H. Förther 7503 (MAF 149194)
B. montanum	**264**	N Morocco: Chefchaouen (area 7)	03.xi.1993	H/W	P. García Murillo *et al.* ST 251/93 (SEV)
	292	Morocco: N High Atlas	05.vii.1997	H/W	S.L. Jury 17456a (E)
	304	N Morocco: Chefchaouen (area 2)	22.vii.1995	H/W	M.A. Mateos *et al.* 6914/95 (SEV)
B. mundii	**283**	South Africa: Natal	05.v.1998	F/C	S. Neves s.n. (E)
B. odontites	**291**	N Tunisia: Kroumirie	11.v.1975	H/W	Davis & Lamond D57628 (RNG)
B. oligactis (= *B. atlanticum*)	**265**	Morocco: High Atlas	12.vii.1987	H/W	S.L. Jury *et al.* 9240 (SEV 127166)
	281	Morocco: Mid Atlas	05.vii.1997	H/W	S.L. Jury 17516 (E)
	298	Morocco: High Atlas	07.vii.1997	H/W	S.L. Jury 17603 (E)
B. plantagineum	**272**	Algeria: Cap Carbon	29.v.1971	H/W	Davis 52959 (RNG)
B. praealtum	**267**	Spain: Lérida	23.viii.1987	H/W	C. Aedo *et al.* ML 286-87 (MA 449973
	288	Spain: Huesca	01.viii.1981	H/W	P. Montserrat *et al.* (JACA 191581)
	289	Spain: Teruel	13.viii.1995	H/W	C. Fabregat & Lopez Udias (JACA 683295)
	308	Spain: Salamanca	02.viii.1983	H/W	J.L.F. Alonso & A. Guillen (MA 518939)
B. ranunculoides	**43**	Europe	13.ix.1994	F/C	S. Neves photo collection (1995)
	181	Europe	23.iii.1998	F/C	S. Neves s.n. (E)
	296	Spain: Burgos	24.vi.1990	H/W	J.A. Alejandre 1078/90 (MA 493699)
	297	Spain: Jaén	10.viii.1982	H/W	C. Soriano (MA 462383)
B. rigidum* ssp. *rigidum	**254**	Spain: Murcia	24.viii.1997	F/W	S. Neves 53 (E)
	261	Spain: Málaga	05.ix.1997	F/W	S. Neves 63 (E)
B. rigidum* ssp. *paniculatum	**70**	Portugal: Beira Litoral	27.vi.1994	F/W	F. Sales & S. Neves [S. Neves 3] (E)
	244	Portugal: Estremadura	10.viii.1997	F/W	S. Neves 31 (E)
B. rotundifolium	**4**	Europe	01.viii.1994	F/C	S. Neves s.n. (E)
B. salicifolium	**29**	Canary Islands	20.ix.1996	F/C	S. Neves s.n. (COI)
	273	Madeira	29.xi.1989	H/W	L. Chilton & N.J. Turland 135 (BM)
	294	Canary Islands: Gran Canaria	19.vi.1995	F/W	M. Gardner & S. Knees SG 5750 (E)

Taxon	Acc. No	Geographical area	Date of collection	F/ H W/ C	Voucher specimen
Bupleurum semicompositum	286	Spain: Ciudad Real	12.v.1992	H/W	S. Cirujano (MA 552469)
B. stellatum	312	Switzerland: Canton du Valais	10.viii.1988	H/W	B. de Retz 88690 (MAF 145370)
B. subspinosum	299	Morocco: High Atlas	21.vii.1976	H/W	C.J. & A.R. Humphries 99 (BM)
B. tenuissimum	233	Portugal: Beira Litoral	30.ix.1996	F/W	S. Neves 22 (E)

9.2.2 General material and equipment

Laboratory equipment:

- fume bench system
- laminar flow workstation
- refrigerator (4°C)
- freezer (-20°C)
- ultracold freezer (-80°C)
- ice machine
- distilled water source
- liquid nitrogen source (self-pressurised dewar vessel)
- autoclave
- balance (precision)
- hot plate stirrer & magnetic stirring-bars
- pH meter
- centrifuge
- vacuum centrifuge
- microtubes heating block(s)
- vortex mixer
- microwave oven
- drying oven
- gel tank electrophoresis apparatus (plus gel rigs or molds and comb bridges)
- UV transilluminator & UV light box
- polaroid camera-system and black & white instant pack films; or digital system (DC 40 camera and computer program Kodak Digital Science 1D, version 1.6, was used)
- thermal cycler (PCR machine) – (Perkin Elmer Gene Amp PCR System 9600, Techne Progene and Techne Cyclogene machines were used)
- automatic sequencer (ABI Prism 377 DNA sequencer was used)
- computer and programs for sequence recording, storage and analysis (see sections 9.2.7 to 9.2.9)

Tools and other material:

- laboratory coats
- latex gloves (boxes)
- rubber and cryogenic gloves
- filter masks, protective glasses/goggles
- first aid box
- fire extinguisher
- sealing/cling film
- aluminium foil
- tweezers, scissors
- spatulas, spoons
- lab markers, labels
- warning and autoclave indicator tapes
- plastic pestles (sterile)
- micropipetters (Gilson Pipetman) & autoclaved tips (10 µl, 20 µl, 200 µl & 1000 µl)
- eppendorf tubes (= microcentrifuge tubes or microtubes), autoclaved (0.2 ml, 0.75 ml & 1.5 ml)
- erlenmeyers and beakers (various sizes)
- thermometer
- various tube racks and storage boxes
- multiwell plates (U-shaped bottom) with cover
- dewar vacuum flask (for liquid nitrogen)
- various bottles, containers or boxes for disposal of chemicals, toxic tips/gloves, broken glass, etc
- ethanol 70% & 100%
- distilled water

Safety:

Be aware that many of the chemicals used in molecular biology are irritant, toxic or extremely toxic, some are proven mutagens and possible carcinogens! Read carefully all the safety guidelines accompanying the different products or equipment in general. Latex gloves should be worn during most of the procedures; changing of gloves is necessary to avoid contamination between different working areas. Wearing of protective glasses (or filters) and gloves (for skin protection) is necessary when working with UV light sources. Toxic solutions or contaminated material (gloves, micropipette tips, etc) should be discharged in appropriate containers for subsequent treatment. See also Issac & Edwards (1998) for general recommendations.

Note:

In the following sections, under material, only chemicals and special equipment is listed; use of micropipetters, tips, eppendorf tubes, scissors, lab markers, etc, are omitted, but size of tubes is referred in the text.

9.2.3 DNA extraction

A) CTAB method

For DNA extraction a modified CTAB method from Doyle & Doyle (1987) was used as described below [CTAB = hexadecyltrimethyl- (or cetyltrimethyl-) ammonium bromide]. Procedure used was the same for fresh and herbarium material.

Material:

- silica gel dried plant material.
- sterile sand.
- plastic pestle (sterile).
- 2x CTAB extraction buffer (see notes below):

  i) 2% w/v CTAB; **ii)** 1.4M NaCl, **iii)** 20mM EDTA (ethylenediaminetetra acetic acid) disodium (addition of NaOH is necessary to dissolve EDTA in water); **iv)** 100 mM Tris-HCl (Trizma hydrochloride); **v)** 1 % PVP-40T (PVP = polyvinylpyrrolidone, Mol. Weigth. 40,000); and **vi)** 0.2 % DTT (dithiothreitol) or 2-mercaptoethanol (= β-mercaptoethanol). Add the DTT or mercaptoethanol only before use.

- isopropanol (= 2-propanol) – keep in the freezer (-20°C).
- 'wet' chloroform: 24 parts of chloroform to 1 part of 1-octanol or IAA (isoamyl alcohol) – [chloroform becomes more hydrophilic with the addition of octanol or IAA, hence being called 'wet'].
- wash buffer: 76 % ethanol + 10mM ammonium acetate.
- TE buffer: 10 mM Tris-HCl + 1mM EDTA disodium; adjust to pH 7.4 with either NaOH or HCl and autoclave.
- heating block (at 65°C).
- centrifuge.
- drying oven.

Notes: The components of the CTAB extraction buffer help to break cell membranes and protect DNA from degradation by endogenous enzymes or secondary metabolites released during lysis. Sodium chloride (NaCl) is a ionic compound that helps the lysis of cell membranes and form complexes with nucleic acids. EDTA is a powerful chelating agent for divalent ions, particurlarly Ca^{2+} and Mg^{2+}, that inactivates metal-dependent nucleases. DTT and β-mercaptoethanol are reducing agents protecting DNA from quinones, disulphides, peroxidases and polyphenol oxidases. PVP-40T complexes secondary metabolites such as tannins, quinones and polyphenols.

Method:

During this procedure work on a fume bench when appropriate, and wear gloves (chloroform and β-mercaptoethanol are toxic by inhalation or skin contact).

1) Cut a piece of leaf (1-2 cm^2) or other dried plant material (add a little more if using herbarium material), and place it inside of a 1.5 ml eppendorf tube. Add a pinch of sterile sand and grind the tissue with a sterile pestle. If material is coriaceous or fibrous, it is recommended to cut it in smaller pieces using a pair of sterile fine scissors – Liquid nitrogen may be used instead of sand, although is obviously more expensive (see step 1-2 in the following method of DNA extraction – section 9.2.3B).

2) Add 200 µl of 2x CTAB (with DTT or β-mercaptoethanol added) and continue to macerate the tissue with the pestle. Add a further 800 µl of CTAB and mix the contents gently. At this stage, the homogenate should be green (for fresh material, but often yellow-brownish when using herbarium material) with small tissue fragments. Incubate the eppendorf tubes at 65°C for 30 min in a heating block (keep a thermometer in it to know the temperature).

3) Remove the tubes from the heating block and leave to cool to room temperature. Add 200 ml of 'wet' chloroform, and mix gently to obtain a momentary single phase. Centrifuge for 2 min at 13,000 rpm [the purpose of this stage is to remove proteins and carbohydrates].

4) Carefully, take out the tubes from the centrifuge, and with a micropipette remove the aqueous upper phase (supernatant) to a new tube (1.5 ml vol.). Centrifuge for 2 min at 13,000 rpm – dispose of chloroform waste in appropriate bottle.

5) Add another 200 µl volume of 'wet' chloroform to the extract, and mix gently to obtain a momentary single phase. Centrifuge for 2 min (13,000 rpm).

6) Remove the supernatant to a clean eppendorf. Add 600 µl of cold (-20°C) isopropanol (just taken from the freezer at the moment of use), and mix gently. Leave for 10-15 min at room temperature [the purpose of this stage is to precipitate nucleic acids].

7) Centrifuge the tubes for 2 min (13,000 rpm) to pellet the nucleic acids.

8) Remove the supernatant with a micropipette – the pellet is now visible and it should be small, thin and clear or whitish (a brownish/ blackish pellet is a warning

of forthcoming problems – it normally means that there is oxidized material and that running of the sample in a gel or PCR amplification may be problematic). Add 1 ml of wash buffer, and agitate to release the pellet from the bottom of the tube, and leave in the refrigerator for at least 1 hour, but preferably overnight (up to 18 hr). It is necessary to leave the pellet in the wash buffer for long enough to remove any residual CTAB, otherwise DNA will not migrate in the gel.

9) Put on the drying oven set for a temperature of 50°C. Take the tubes out from the refrigerator, and centrifuge them for 2 min to pellet the nucleic acids. Carefully, remove the supernatant. Invert the tubes over paper tissue in a small tray (make sure that the pellet does not slip out of the tube), and place it in a drying oven for c. 10 min (50°C).

10) Once the pellets are dry, dissolve with c. 75 µl of TE. Store in the refrigerator for short term, or move to a -20°C freezer for long term storage.

B) DNA extraction with DNeasy plant mini kit

DNeasy plant mini kit (Qiagen Ltd., UK) allows fast DNA extraction from plant material (fresh or herbarium) producing good yields of purified DNA of high molecular weight. As it uses a silica-gel membrane with selective binding properties (see also section 9.2.6), is capable, in principle, of removing inhibitors of PCR or other enzimatic reactions.

The DNeasy kit was used only for 6 samples: **a)** 4 samples which DNA extraction had previously failed (all herbarium material): *Bupleurum album* (Acc. No 269),1 *B. barceloi* (Acc. No 295), *B. foliosum* (Acc. No 275), and *B. subspinosum* (Acc. No 299); **b)** 1 sample of fresh material (*Glia prolifera*, Acc. No 284) where PCR amplification had failed, in spite of having a good yield of DNA extract; **c)** 1 sample which DNA extraction had not yet been attempted (*B. canescens*, Acc. No 301).

Material:

- DNeasy™ plant mini kit – Qiagen Cat. No 69103 (for 20 samples) or No 69104 (for 50 samples). This kit includes *DNesay spin columns*, *QIAshredder spin columns*, *collection tubes* (2 ml), and 5 different *buffers*: AP1 (lysis buffer), AP2 (precipitation buffer), AP3 (binding

buffer), AW (concentrated - wash buffer), and AE (elution buffer) – all which are stored at room temperature; it also includes RNase A that should be stored at 4°C.

- liquid nitrogen.
- dewar vacuum flask.
- plastic pestle (sterile).
- vortex mixer.
- heating block.
- centrifuge.

Method:

The following is a brief description of the protocol, for further explanation see user's handbook included with the DNeasy plant mini kit (minor changes to the protocol were recommended by Dr Achariya Rangsiruji). Although not strictly required in the Qiagen protocol, liquid nitrogen was used to grind the plant tissue, as it is probably the best method to reduce the material to a powder, which is important to obtain a good yield of DNA using this extraction kit.

1) Decant into a dewar vacuum flask c. 1/2 litre of liquid nitrogen from your source (self-pressurised dewar vessel) – Follow safety instructions; wear cryogenic gloves and protective glasses while decanting the liquid nitrogen as it can cause burns.

2) Cut a piece of leaf (1-2 cm^2) or other plant material (add a little more if using herbarium material) and place it inside of a 1.5 ml eppendorf [i.e. the same amount of material used for the CTAB method, but in this case, as we use liquid nitrogen, fresh material can be directly used, no need of drying with silica gel]. Put some liquid nitrogen into a small beaker and then submerge the eppendorf (closed) into the liquid nitrogen to freeze the material; you can also pour some liquid nitrogen inside the eppendorf and leave to evaporate – work with a single sample at a time; liquid nitrogen tends to spill over ('boil') and adjacent tubes may be contaminated. Open the eppendorf and grind the tissue with a (plastic) pestle to a fine powder. Continue to freeze the sample with liquid nitrogen until completely ground – do not allow it to thaw.

3) After the sample has been reduced to a fine powder, add 400 µl of buffer AP1 (lysis buffer) and also 20 µl of RNase A [RNase is generally not necessary when working with herbarium material, as RNA is normally already degraded]. Vortex the sample vigorously (no tissue clumps should be seen). Do not mix AP1 buffer and RNase prior to use.

4) Incubate the tubes in a heating block for 10 min at 65°C (30 min for herbarium material). Mix 2-3 times during incubation by inverting the tubes.

5) Add 130 µl of buffer AP2 to each tube, mix and cool in ice for 10 min. Centrifuge for 5 min at maximum speed (13,000 rpm).

6) Place QIAshredder spin column in a collection tube (2 ml vol.), and apply supernatant into the spin column. Centrifuge for 2 min at maximum speed.

7) Transfer the fluid in the collection tube to a 1.5 ml eppendorf tube (if there is a small pellet of cell-debris at the bottom, do not disturb it while pipetting). Determine approximately the volume of the fluid recovered (necessary for the next step) – Typically the volume is c. 450 µl, but it might be less.

8) Add 0.5 volume of buffer AP3 and 1 volume of 96-100% ethanol to the eppendorf and mix by pipetting.

9) Apply 650 µl of the mixture (total volume normally is 900-1100 µl) from step 8, including any precipitate that may have formed, onto a DNeasy mini spin column sitting on a collection tube. Centrifuge for 1 min at ≥ 8,000 rpm. Discard the fluid in the collection tube. Apply remaining sample to the column and centrifuge for another minute. Discard liquid and collection tube.

10) Place DNeasy column in a new collection tube, and add 500 µl of buffer AW (with added ethanol) onto the DNeasy column. Centrifuge for 1 min at ≥ 8,000 rpm. Discard collected liquid, but reuse the collection tube in the next step.

11) Add 500 µl of buffer AW to DNeasy column and centrifuge for 2 min at maximum speed (13,000-13,200 rpm) to dry the column membrane.

12) Place DNeasy column in a 1.5 ml eppendorf. Pipet 50 µl of preheated (65°C) buffer AE (elution buffer) onto the DNeasy column membrane. Leave to stand for 1 min, and then centrifuge at ≥ 8,000 rpm for 1 min to elute the DNA.

13) Repeat elution as described (step 12); a new eppendorf may be used to prevent dilution of the first eluate. Store DNA extracts in the refrigerator (4°C) for short term, or move to a freezer (-20°C) for long term storage.

9.2.4 Gel electrophoresis

Once the extraction samples are ready, it will be necessary to check that DNA extraction was successful. To do this, a small volume of the extract (5 µl) is run in an agarose gel under an electrical field.

Material:

- agarose (wide range/standard 3:1 - Sigma A-7431).
- TBE buffer (used at 0.5x or 1x concentration):

  For 1 litre 5x TBE buffer (stock): 27.5 g boric acid + 54 g Trizma (or Tris) base [2-amino-2-(hydroxymethyl)-1,3-propanediol] + 20 ml of 0.5M EDTA pH 8.0 [EDTA will only dissolve in water with the addition of NaOH].
- ethidium bromide solution: dissolve 200 mg ethidium bromide (Sigma E-8751) in 20 ml of distilled water, and store in a light-proof container at 4°C. Ethidium bromide is a nucleic acid intercalating agent, it specifically binds double-stranded DNA (or RNA), and is fluorescent when irradiated with UV light. Be extremely careful while handling ethidium bromide, it is very toxic and a potent mutagen and likely carcinogen. Wear gloves and mask when weighing the powder, and always wear gloves when working with the solutions – [store solution at 4°C].
- loading buffer (gel loading solution – Sigma G-2526) – this solution includes a dye, bromphenol blue, that helps to visualize the advancement of the samples in the gel – [store at room temperature].
- ladder (weight markers): Lambda (λ) DNA *Hin*dIII digest (Sigma D-9780); or 123 bp DNA ladder (Sigma D-5042) – [keep in the freezer at -20°C].
- ladder buffer (to use with 123 bp DNA ladder): 10mM Tris-HCl + 50mM NacCl + 0.1mM EDTA – [store at room temperature].
- gel tank electrophoresis apparatus plus gel rig and comb.
- UV transilluminator plus UV light box.
- polaroid camera-system and black & white instant pack films; or digital system (DC 40 camera and computer program Kodak Digital Science 1D, version 1.6, was used)

Method:

1) Preparation of gel:

- *Small gel*: 50 ml of 0.5x/1x TBE buffer + 0.75 g of agarose (1.5% agarose) + 0.5 µl of ethidium bromide solution (taken from the refrigerator at the moment of use).
- *Large gel*: 80 ml of 0.5x/1x TBE buffer + 1.2 g of agarose + 0.8 µl of ethidium bromide.

a) Cover the ends of the gel rig (mold) with sealing tape, and place the comb bridge (that creates the wells in the gel) in one of the ends; make sure that the gel rig is placed horizontally.

b) Put the agarose and the volume of TBE buffer in an erlenmeyer (concentration of the TBE buffer in the gel should be approximately the same than that of the TBE buffer in the gel tank). Cover the erlenmeyer with cling film (to avoid the solution spilling over), and make a small hole on the top. Heat in the microwave for c. 1 min (max. power), or until agarose is completely dissolved. Leave the solution to cool a little, and add the ethidium bromide (be careful! – see notes above).

c) Pour the agarose solution (with ethidium bromide) onto the gel rig. If any bubbles are formed, 'punch' them with a micropipette tip. Leave the gel to cool at room temperature until is firm (10-20 min); after, move it to the refrigerator for another 5-10 min (cover loosely with cling film to minimize contamination of the fridge). After gel is set, carefully remove the comb and the tape, and place the gel rig inside the electrophoresis tank that should already contain the TBE buffer – check that the gel is completely submersed by TBE; add more if necessary.

2) Preparing samples for loading:

To check the extract of *total DNA*, the following ladder solution is used: 1 part of lambda (λ) DNA *Hin*dIII digest, 3 parts of loading buffer, and 6 parts of distilled water. After preparation, store in the freezer at -20°C.

To check *PCR products* (before and after purification) the following ladder solution is used: 1 part of 123 bp DNA ladder, 3 parts of loading buffer, and 6 parts of ladder buffer. After preparation, store in the freezer at -20°C. *Note*: this solution

needs to be heated to 65°C before electrophoresis (place in the heating block and leave for 5 min).

Prepare the DNA samples in a multiwell plate. Mix 5 µl of 'DNA' extract and 2µl of loading buffer for each sample to test. For verification of PCR success, 2 µl of PCR product and 2µl of loading buffer are used. To check purified PCR products only 1µl of DNA is added to 1µl of loading buffer.

3) Loading the gel: Transfer (load) each of the samples into a well of the gel that should already be placed inside of the gel tank. Load also the ladder: 5 µl of λDNA *Hin*dIII solution into one well or two (one in each of the sides of the gel) – one ladder is enough, but two ladders are recommended if many samples are run or if it is a large gel. When running PCR products the ladder used is the '123 bp' DNA.

4) Electrophoresis of the gel: Run the gel at 60V (for 1.5-2 hr) to 80V (for 1-1.5 hr). Speed of the movement of the molecules increases with increase of voltage, but bands may lose definition.

5) Visualization of the gel and recording of image: Move the gel to the UV transilluminator and photograph. A polaroid camera can be used and the printed photograph will be obtained in c. 2 min. But the image of the gel can be digitally recorded by a DC 40 camera and visualized using the computer program Kodak Digital Science 1D (version 1.6). In this case, the image of the gel can be saved in a computer file and printed when required. The quality of the polaroid image is better, but for the current work the image recorded by the DC camera had the necessary quality (in the long term, it is cheaper to use this camera as there is no need to buy films). Fig. 9.1 (below) shows examples of gels obtained after running: **A)** DNA extracts; **B)** PCR products; and **C)** purified PCR products.

6) Interpreting band patterning in the gel.

a) *DNA extracts*: In Fig. 9.1A, the 6 different samples show different patterns of distribution of DNA in the gel. Samples 3 & 6 were obtained from fresh material, and 1, 2, 4 & 5 from herbarium specimens; in general, concentration of total DNA is higher in extracts from fresh material. Samples 2, 3, 4 & 5 clearly show a band of high molecular weight representing total DNA. Sample 6 also shows high

concentration of total DNA, but the band is obscured by a strong smear of degraded DNA (fragments of many sizes). Any of these samples (2-6) show good yield of total DNA for subsequent PCR amplification (only a minute quantity is really necessary). Sample 1, however, shows only a light smear and amplification may be more problematic. Nevertheless, in this particular example, PCR amplification was successful for all the samples. Therefore, if there is at least a light smear of DNA in the region of high molecular weight, PCR amplification should be attempted.

b) *PCR products*: Fig. 9.1B shows the results of the PCR (see next section) of 9 samples. PCR failed only in one of the samples (no. 3). The bands are more or less aligned in the gel, indicating that the amplified fragments have a similar molecular weight (which is often the case in the ITS region – it is highly conserved in length). However, sample no. 1 seems to be slightly 'lighter' as has moved a little faster. In fact, this is a sample of *Bupleurum acutifolium* Boiss. (Portuguese population) that sequencing revealed to have a considerable deletion (35 bp) in ITS 1 (see section 9.4 for discussion).

Samples where PCR was successful need to be purified (see section 9.2.6), and run again in a gel for verification of purity. Therefore, the main difference between the bands of the samples run after PCR, and after they have been purified is that the latter should look 'cleaner' – i.e. the smear of other PCR products (especially the smaller fragments) should disappear or be considerably reduced – compare Fig. 9.1B & C (bands in the gel of Fig. 9.1C have moved further down the gel simply because the gel was left to run for longer time).

The ladder of 123 bp DNA ('**La**' in Fig. 9.1B & C) is used to estimate the concentration of DNA (i.e. PCR product), information that is required for effective sequencing. The different bands in the ladder have known concentrations: the lightest band (123 bp), moving faster, has a DNA concentration of 30 ng/µl, the next band following it (246 bp) has 10 ng/µl of DNA. To estimate the concentration of PCR products, brightness of the bands are compared. The bands in the ladder are more clearly visible in Fig. 9.1B, because the image was scanned from a polaroid print, while Fig. 9.1C was obtained from printed image recorded by a PC 40 camera (Kodak Digital Science). As mentioned before, the quality of image in the latter is

inferior, but a good printed image is not really necessary as DNA concentration can be estimated directly by looking to the gel under UV light.

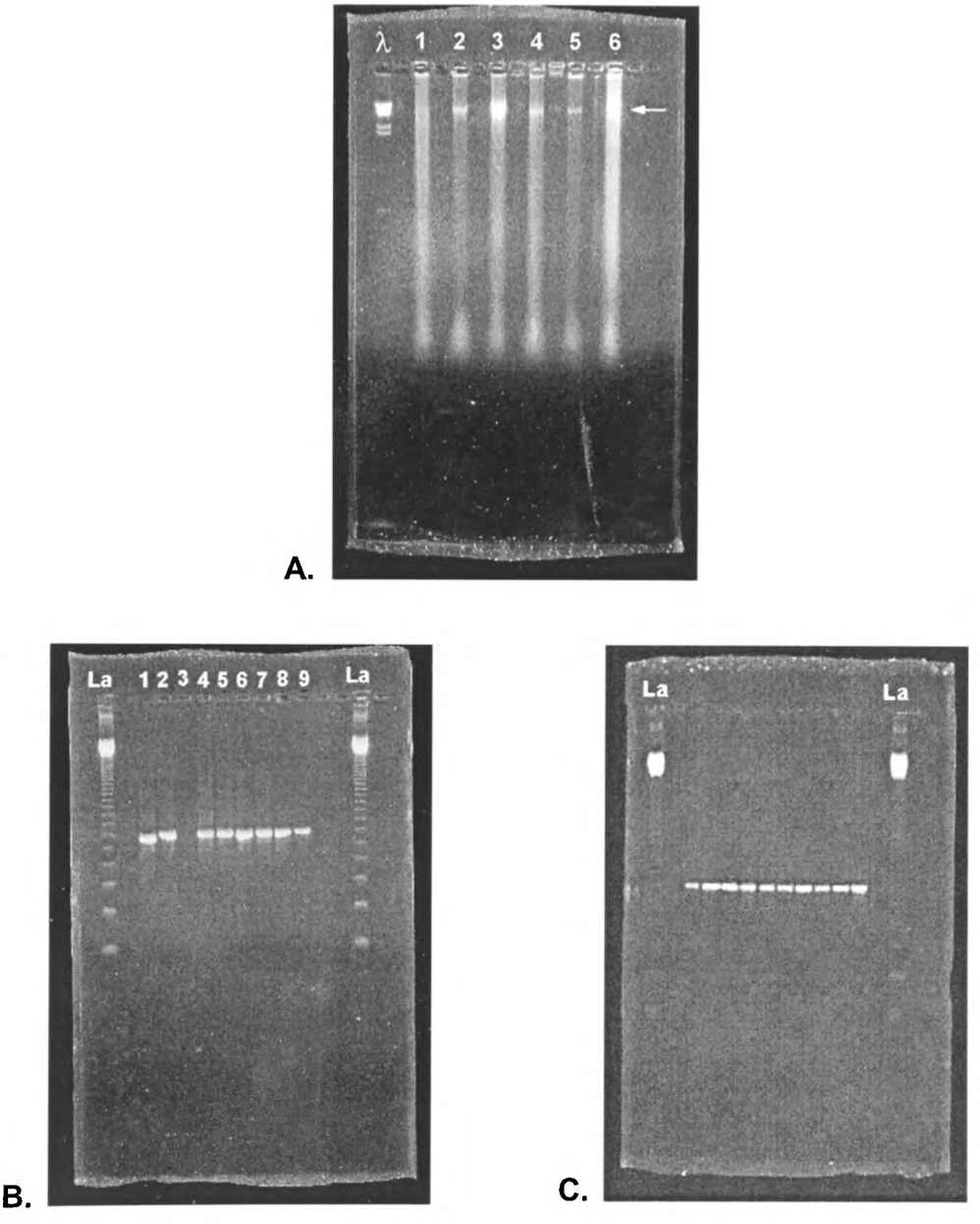


Fig. 9.1 - Gel images after electrophoresis. A. DNA extracts (the arrow indicates the band of total DNA; the smear represents degraded DNA). **B.** PCR products (notice that PCR amplification failed in sample 3). **C.** Purified PCR products. Weight markers: λ = lambda DNA *Hin*dIII digest; **La =** 123 bp DNA ladder.

9.2.5 Amplification (PCR) of the gene (ITS region)

The *polymerase chain reaction* (PCR) allows the exponential amplification of specific DNA segments from which at least part of their sequence is already known [PCR can also be used to amplify at random unknown segments of DNA – see discussion on RAPDs, in section 9.1]. The known sequence is used to design two synthetic oligonucleotides (**primers**) that are complementary to DNA on opposite strands in the flanking regions of the segment to be amplified. These primers will bind to single-stranded DNA and act as the starting points for *in vitro* duplication of DNA. A DNA polymerase and deoxynucleoside triphosphates (dNTPs) are required in the reaction. In each cycle of duplication, the double-stranded DNA needs to be separated into single strands, which is done by heating.

The principle of PCR has been known for many years, but the great breakthrough came with the isolation of a thermostable DNA polymerase, the *Taq* polymerase, that is not denaturated with the repeated heat treatments – the *Taq* enzyme is obtained from the thermophilic bacterium, *Thermus aquaticus*. Each cycle of the PCR consists of three main phases (see e.g. Griffiths *et al.*, 1996, or Aert *et al.*, 1998; and for detailed discussion of PCR, see Palumbi, 1996):

1) Denaturation: A brief heat treatment separates the double helix of genomic DNA into single strands. In general, the temperature used is 94°C during 30 sec – the *Taq* polymerase is resistant to high temperatures, but after several cycles of repeated heating it will lose activity if denaturation time is longer. A longer first denaturation step has been recommended, because initial full genomic denaturation is critical.

2) Annealing: The temperature is lowered (40-60°C for about 60 sec), and the primers, that are in great excess, will bind to their complementary sequences in the single-stranded DNA.

3) Extension: The tubes are then heated up to 72°C; this is the optimal temperature for the *Taq* polymerase to work. The enzyme will start at the 3' end of each primer and, using the single strands as templates, will synthesize new DNA in the 5' → 3' direction. The extension time will depend on the length of the segment to be amplified. The *Taq* polymerase can add thousands of bases per minute, therefore,

for PCR products under 500 base pairs (bp), 30 sec are enough to complete duplication. For PCR products between 500 and 1500 bp, 60 sec will be required, and 90 sec for longer segments [ITS region is 600-700 bp long, so 1 min of extension is recommended for effective duplication].

For effective PCR amplification, 20 to 30 cycles of the above reactions are required. The number of cycles can be increased up to 40 cycles if the initial concentration of DNA is very low.

In the case of the **ITS region** [see section 9.1.1B - 6) DNA sequencing], the **primers** used are based on those originally designed for fungal ribosomal DNA (White *et al.*, 1990). **'ITS 5'** is located (see Fig. 9.2) at the end (5'→3') of the 18S nuclear ribosomal DNA (nrDNA) – ['ITS 5P' differs in two nucleotides from the original primer]. **'ITS 4'** is located at the start of the 26S nrDNA (25S or 28S in other eukaryotic organisms). These primers are located in coding regions that are evolutionary conservative. However, it might be necessary to correct the sequence of the primer for particular plant groups, in order to increase the stability of primer annealing; this will be possible after sequencing of one (preferably 2) of the samples. There are also alternative primers for ITS sequencing (White *et al.*, 1990), all of which have binding sites within the flanking coding regions. If the primers fail to anneal (one of the possible reasons of failure of PCR), other primers can be used, such as: 'ITS 1' (instead of 'ITS 5'), and 'ITS 8' (instead of 'ITS 4').

Material:

- distilled water (dH_2O).
- 10x *Taq* polymerase buffer (*): 15 mM $MgCl_2$ +10x NH_4 buffer [160 mM $(NH_4)_2SO_4$ + 670 mM Tris-HCl + 0.1% Tween-20].
- dNTPs (*): master mix of dATP, dCTP, dGTP, dTTP, 12.5 mM solutions (Bioline, UK – Catalogue No S40200).
- primers (*) (Oswell DNA Service, University of Southampton, UK):
  'ITS 5P' – GGA AGG AGA AGT CGT AAC AAG G [forward primer]
  'ITS 4' – TCC TCC GCT TAT TGA TAT GC [reverse primer]
- *Taq* polymerase (*) (5 units/µl): Biotac™ polymerase, (Bioline, UK – Cat. No M98501B) – Unit is defined as the amount of enzyme that incorporates 50 nmoles of dNTPs into acid-insoluble form in 30 min at 72°C under defined assay conditions (see Bioline catalogue 1998/99).

- DNA extract (concentration c. 2 ng/µl) – [stored at 4°C for sort term use, or at -20°C for long term use].
- centrifuge.
- thermal cycler (PCR machine) – use appropriate volume of tubes (0.2 ml for Perkin-Elmer PCR machine, and 0.75 ml for either Techne Cyclogene or Progene PCR machines).

(*) Store at -20°C, in a constant temperature freezer.

Method:

It is critical to work under sterile conditions while preparing the tubes for PCR, any minute amount of contaminant DNA may be amplified, not only from other plant material used in the same laboratory, but also (for certain genes) from human DNA! Use of gloves is indispensable, and tubes should only be open inside of the flow cabinet. Verify if there are enough autoclaved tips to prepare the samples.

1) Switch on the laminar flow workstation, and carefully wipe it down with 70% ethanol. Place on the side of the flow cabinet a basin with ice. Take out from the freezer the *Taq* buffer, dNTP's (deoxynucleotides), primers and DNA extracts, and leave to defrost inside the flow cabinet. Start labelling the microtubes. After thawing, keep the tubes with DNA and the other solutions in the ice. The enzyme (*Taq* polymerase) is taken from the freezer only at the moment of use.

2) Pipette the following volumes to each of the reaction tubes. The order of addition can be changed, but it is advisable to add the enzyme and the DNA just at the end. The volume used of the enzyme is minute (0.2 µl), but is still visible to the naked eye – if you do not see anything in the tip, you should try to pipette again.

For each sample/ reaction:

- dH_2O	31.8 µl
- 10x Taq buffer	5 µl
- dNTPs (10 mM)	1 µl
- primer 1 [5p or 4] (10 µM).....	5 µl
- primer 2 [4 or 5p] (10 µM)	5 µl
- DNA extract (c. 2 ng/µl of DNA)	2 µl
- Taq polymerase (5 units/µl)	0.2 µl
Total volume:	50 µl

Notes:

a) The use of the same amount/concentration of both forward and reverse primers results in a double-stranded PCR product (if annealing of both primers is efficient). If primers are present in different concentrations a mixture of single- and double-stranded DNA will be produced (ethidium bromide is specific for double-stranded nucleic acids, so only the latter can be detected during electrophoresis).

b) The volume of DNA extract added may have to be changed – but always try the PCR first. It may be necessary to increase the volume of DNA extract added, if its concentration is very low. This was the case for one of the samples extracted from herbarium material (*Bupleurum album* Maire, Acc. No 269); PCR was only effective when 4 µl of DNA extract were used (instead of 2 µl). In another sample (*B. longifolium* L., Acc. No 310), it was the opposite, PCR was only successful when the volume of DNA extract was reduced to 1 µl. The problem in this case probably was the presence of compounds that inhibited the PCR reaction, and which concentration was reduced by decreasing the volume used.

3) After adding all the components to the reaction tubes, flick the base of the tubes tubes to mix the contents. Centrifuge the tubes till reaching the speed of c. 9,000 rpm (only a few seconds). Place now the tubes in the PCR machine (thermal cycler), select the appropriate program and run for the time required (normally for 3-4 hours).

Basic program used for ITS amplification:

- Initial denaturation: 94°C - 3 min
- Cycles (30 times):
  - 94°C - 1 min (*denaturation*)
  - 55°C - 1 min (*annealing*)
  - 72°C - 1.5 min (*extension*)
- Finalizing primer extension: 72°C - 5 min
- End of reaction: keep at 4°C

Electrophoresis on gel: After the PCR program is completed, check PCR success by running 2 µl of PCR product and 2 µl of loading buffer on a 1.5% agarose

gel. Use 3 µl of 123 bp ladder (heat at 65°C for 5 min before use) to estimate concentration of DNA (for explanation see section 9.2.4).

9.2.6 Purification of PCR products

Samples where PCR was successful need to be purified to remove excess of primers, dNTPs and other impurities. It is essential to remove any remaining primers in the PCR products (in this case 'ITS 5P & 4') before sequencing. If more than one type of primer is present in the reaction, more than one sequence will be produced, which will make them unreadable as the different sequences and their fluorescent peaks will overlap to each other (see section 9.2.7A & C). Also, dNTPs left from PCR will affect the balance on the components of the sequencing reaction.

Qiagen Ltd. (UK) developed a kit that allows easy and efficient purification of PCR products. Each PCR product is applied to a spin column that contains a silica-gel membrane with selective binding properties. DNA (single- or double-stranded, ≥ 100 bp) binds to the membrane in the presence of high salt concentration, while contaminants pass through the column. After impurities are washed away, the DNA is eluted with a Tris buffer or water.

Material:

- QIAquick PCR purification kit – Qiagen Cat. No 28104 (for 50 samples) or 28106 (for 250 samples). This kit includes *spin columns*, *collection tubes* and 3 different *buffers*: PB, PE (concentrated - before use add 24 ml of ethanol) and EB (elution buffer: 10 mM Tris-HCl, pH 8.5) – [store at room temperature].
- centrifuge.

Method:

Work on a fume bench and wear gloves. The following is the protocol recommended by Qiagen Ltd.

1) Add 5 volumes of buffer PB to 1 volume of PCR product – [In this case we have 50 µl of PCR product, so we need to add 250 µl of PB buffer (the 2µl of PCR product that was run on gel is negligible). If the microtubes cannot accommodate the

total volume to be added (220 µl is the maximum volume of 0.2 ml tubes), transfere PCR products to larger tubes (vol. 0.6 or 0.75 ml)].

2) Place QIAquick spin column in a 2 ml collection tube, and carefully pipette the sample into the column.

3) Centrifuge the tubes with the spin columns at 13,000 rpm for 30 sec. Discard the solution that passed to the collection tube – DNA is now bound to the silica-gel membrane of the spin column.

4) Apply 600 µl of PE buffer (diluted with ethanol) into the spin columns and centrifuge the tubes at 13,000 rpm for 30 sec. Discard flow that passed through, and repeat with another 600 µl of PE buffer – this buffer washes impurities of the DNA.

5) Place each spin column into a 1.5 ml eppendorf tube [cut the lids off, but keep them aside]. Carefully apply c. 40 µl of EB buffer to the centre of the membrane of the spin column to insure that is completely covered by the buffer (use a smaller volume, 35 or 30 µl, of EB buffer if concentration of PCR product is low). Allow to stand for 1 minute, and then centrifuge at 13,000 rpm for 1 min. The EB buffer has a low salt concentration, so DNA will be eluted into the eppendorf. Discard spin columns, and put the lids back onto the tubes. Store the tubes at 4°C for short term use, or place them in a freezer at -20°C.

Electrophoresis on gel: Check purity of PCR products by running 1 µl of DNA sample plus 1 µl of loading buffer on a 1.5% agarose gel. Use 3 µl of 123 bp ladder (heated at 65°C for 5 min) to estimate DNA concentration (for explanation see section 9.2.4).

9.2.7 ITS sequencing

A) Cycle sequencing using dideoxynucleotides

Cycle sequencing is based on Sanger dideoxy sequencing method (Sanger *et al.*, 1977), where a mixture of deoxynucleosides triphosphates (dNTPs) and dideoxynucleosides triphosphates (ddNTPs) is used. Each dideoxynucleotide lacks a hydroxyl group (3'OH) on its deoxyribose, and cannot form a phosphodiester bond

with next incoming dNTP. DNA replication starts at defined primer sites in the presence of a DNA polymerase, but each time that a ddNTP is incorporated instead of a normal dNTP, chain elongation is blocked [hence ddNTPs being called 'terminators']. Several cycles of the reaction (using the PCR principle: denaturation, annealing, extension - see section 9.2.5) are performed and the result is a mixture of fragments of many sizes (potentially as many as the number of bases present in the sequence of DNA we wish to find). Each dideoxynucleotide has attached a marker, either a radioisotope (for manual sequencing) or a fluorescent dye, with a different colour emission for each of the 4 nucleotides (automated sequencing) – there is also the possibility of labelling the deoxynucleotides or the primer. The labelled fragments are then separated by electrophoresis in a polyacrylamide gel (see section 9.2.7C) which allows separation of fragments that differ in length by a single base – see e.g. Griffiths *et al.* (1996); or Hillis *et al.* (1996c)].

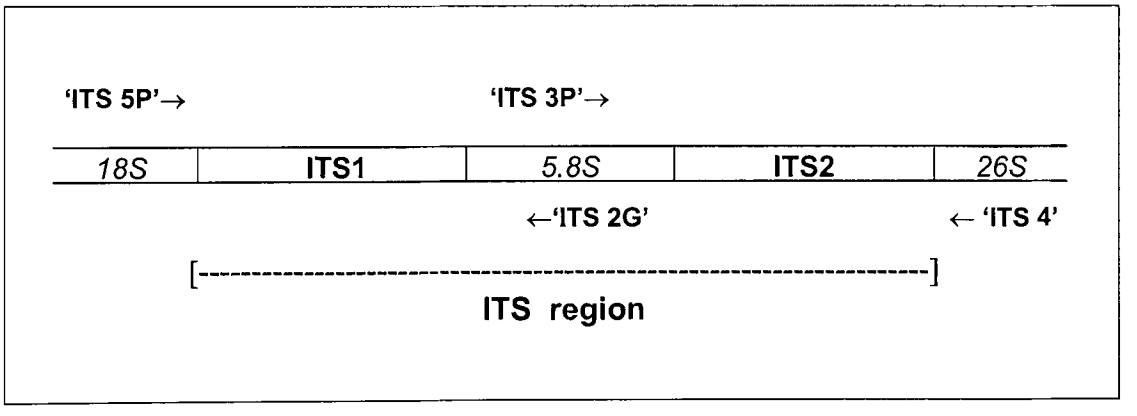


Fig. 9.2 - The ITS region of nuclear ribosomal DNA: The internal transcribed spacers (ITS1 and ITS2) and the coding region of the 5.8S. Notice that the binding sites of primers ('ITS 5P', 'ITS 4', 'ITS 3P', & 'ITS 2G') used to sequence ITS are located in coding (conservative) regions (18S, 5.8S and 26S of nuclear ribosomal DNA) – based on White *et al.* (1990) and Baldwin (1992). [See also Fig. 9.1 in section 9.1.1B - DNA sequencing].

Although we can obtain the sequence of interest using a single primer ['ITS 5' or 'ITS 5P' can 'read' (provide) the whole ITS region: spacer ITS1, 5.8S, and spacer ITS2], it is highly advisable to use, at least, a second primer ('ITS 4') that reads the sequence from the opposite direction, on the complementary strand. In the

case of the ITS region, we can use two additional primers (primers 'ITS 2' & 'ITS 3' – White *et al.*, 1990) which binding sites are located within the conservative region of the 5.8S nrDNA. These primers are often called 'internal primers'; primer 'ITS 2' (or 'ITS 2G' as used here) reads the complementary sequence of the spacer ITS1; primer 'ITS 3' (or 'ITS 3P') reads the sequence of the spacer ITS2 (see Fig. 9.2).

PE Applied Biosystems (The Perkin-Elmer Corporation, USA) has developed a kit that simplifies the preparation of samples for cycle sequencing using the terminator reaction and optimizes the reading of the sequences by an automated sequencer (see section 9.2.7D).

Material:

- DNA sequencing kit (*): dRhodamine terminator cycle sequencing ready reaction (Part No. 403042). ABI Prism™ - Perkin Elmer Applied Biosystems (Warrington, Cheshire, UK). This kit includes a *terminator ready reaction mix* that contains dNTPs, ddNTPs (dRhodamine dye terminators), Ampli *Taq*® DNA polymerase FS (*Taq* FS), $MgCl_2$, and Tris-HCl buffer (pH 9), and also a *DNA control sample* [pGEM®-3Zf(+), double-stranded DNA template] and a *control primer* (-21 M13).

  Note: The terminator reaction mix is light-sensitive, so keep it in the dark (cover the tube with aluminium foil, and try to minimize light-exposure while pipetting the samples).

- primers (*) (Oswell DNA Service, University of Southampton, UK):

  'ITS 5P' – GGA AGG AGA AGT CGT AAC AAG G
  'ITS 4' – TCC TCC GCT TAT TGA TAT GC
  'ITS 3P' – GCA TCG ATG AAG AAC GTA GC
  'ITS 2G' – GTG ACG CCC AGG CAG ACG T

- distilled water (dH_2O).
- purified DNA (PCR product: ITS region in this case) - [keep at 4°C for short term use or at -20°C for long term storage].
- centrifuge.
- thermal cycler: Perkin Elmer Gene Amp PCR System 9600.

(*) Store at -20°C, in a constant temperature freezer.

Method:

As PCR is involved, much care needs to be taken to work under sterile conditions. Work in a flow cabinet (clean it with ethanol before starting to prepare the samples), and wear gloves. Check if there are enough autoclaved tips to prepare the samples. Put a basin with ice beside the flow cabinet.

1) Remove primers, terminator ready reaction mix, and purified DNA from the freezer, and leave to defrost inside the flow cabinet; after thawing keep the tubes in the ice. Start labelling the tubes. For each sample to sequence, prepare 4 reaction tubes, one for each of the primers ('ITS 5P, 4, 3P, 2G'), and also an extra tube for control. For the primers of 'long run' ('ITS 5P & 4'), i.e. the ones that allow the sequencing of the whole ITS region, we need to add at least 80 ng/µl of DNA; for the primers of 'short run' ('ITS 3P & 2G'), c. 40 ng/µl are required. See section 9.2.4 for explanation on how to estimate DNA concentration of purified PCR products on gel. For bright bands of PCR product (see Fig. 9.1), 2 µl of DNA were added for the long run reactions, and 1 µl of DNA for the short run. Adjust volume of distilled water to complete 20 µl volume per tube.

	Per reaction	**Control tube**
- dH_2O	9-12 µl	8 µl
- terminator ready reaction mix	6 µl	6 µl
- primer 3.2 µM [= 3.2 pmol/µl]	1 µl	4 µl (*)
- purified DNA (10-30 ng/µl)	1-4 µl	2 µl (*)
Total volume:	20 µl	20 µl

(*) Concentration of control primer = 0.8 pmol/µl, and of control DNA = 0.2 µg/µl

2) After adding all the components of the reaction, flick the tubes to mix the contents, and centrifuge briefly (a few seconds). Place the tubes in the PCR machine and select the appropriate program, and leave to run for the time required.

Program used for cycle sequencing:

- Cycles (25 times):
  - 96°C - 10 sec (*denaturation*)
  - 50°C - 5 sec (*annealing*)
  - 60°C - 4 min (*extension*)
- End of reaction: keep at 4°C.

B) Ethanol precipitation

After cycle sequencing, unincorporated dye terminators (dideoxynucleotides labelled with fluorescent dye) are removed by precipitation of DNA. It is essential to remove fluorescent terminators, if they are not efficiently removed, there will be large terminator peaks (background peaks) in the electropherograms which makes impossible to read the sequences – see sections, 9.2.7C & D. The following is the basic protocol recommended by Perkin Elmer to be used with Ampli*Taq* DNA polymerase FS dye terminator chemistry (sequencing kit).

Material:

- solution of 70% ethanol and 0.5 mM $MgCl_2$
- centrifuge.
- vacuum centrifuge.

Method:

Work on a fume bench and wear gloves.

1) Transfer the sequencing products (20µl) to 0.75 ml microtubes.

2) Add 74 µl of a solution of 70% ethanol and 0.5 mM $MgCl_2$. Close the tubes and vortex briefly.

3) Leave the tubes at room temperature for 10 to 15 minutes to precipitate the extension products. A precipitation time of less than 5 min will result in loss of very short extension products.

4) Place the tubes in the centrifuge and mark the orientation of the tube [place all tubes with the hinge of the lid facing the centre of the centrifuge; the DNA pellet will precipitate at the bottom of the tube on the opposite side]. Centrifuge for 20 min at maximum speed (13,000-13,100 rpm).

5) Immediately after the end of centrifugation, start removing, very carefully, the supernatant [The pellet of DNA is normally not visible, but it should be placed, not exactly at the bottom of the tube, but on the side wall; so we can touch the bottom with the pipette tip].

6) Check if there are any drops of supernatant in the tubes. If so, place the tubes in the centrifuge with the same orientation as step 4, and centrifuge for about 5-

10 sec. Remove the tubes, and carefully aspirate the remaining supernatant [often a small drop of supernatant (liquid) can be seen on the side of the tube where the pellet of the DNA is placed; to remove it flick the tube lightly to make the drop to fall to the bottom, there it can be 'safely' aspirated with a pipette].

7) After removing all supernatant, dry the tubes in a vacuum centrifuge for 1 to 3 min. Close the tubes and keep refrigerate at 4°C for short term use or place in a freezer for long term storage (it may be necessary to wait some time before the samples can be run in a automated sequencer).

D) Automated Sequencing

This stage of the sequencing technique was carried out by Nicola Preston, responsible for the automated sequencing facility at ICMB (Institute of Cell & Molecular Biology, University of Edinburgh). An automated sequencer is an expensive instrument, and to be cost effective, its use is centralised to benefit many groups/individuals from a single or even different institutions.

The Perkin Elmer ABI Prism™ 377 DNA sequencer was used. This instrument is capable of determining base sequence, fragment size and quantity of fluorescent dye labeled nucleotide fragments. It includes an electrophoresis system with a vertical gel cassette for a polyacrylamide gel. A Power Machintosh® computer is also necessary to install the software of the ABI Prism 377 DNA sequencer.

After ethanol precipitation (previous section), each tube should have a dry pellet of labelled DNA fragments (with fluorescent dyes) at its bottom. For automated sequencing, DNA needs to be resuspended with a loading buffer [6-9 µl per tube of 5:1 deionized formamide: 25 mM EDTA with 50 mg/ml blue dextran]. The mixture is then loaded into a lane of the (vertical) polyacrylamide gel (it is possible to analyse up to 36 lanes simultaneously on one gel). Under the electric field, the dye-labelled DNA fragments move down the gel and separate according to size (fragments differing in size by a single base can be distinguished). At a lower level of the gel, the fragments pass through a region where a laser ray continuously scan across the gel. The laser excites the fluorescent dyes of the DNA fragments,

which then emit light (at a specific wavelength for each dye – one per nucleotide), which is collected by a spectrograph and recorded by a special camera; all 4 types of fluorescent emissions can be detected with one pass of the laser. The data collection software records the light intensities, process them, and produces electropherograms that can be visualized by programs of sequencing analysis (see next section).

E) Establishing the sequence for each sample

For each reaction tube, two type of sequence data files are produced by automatic sequencing. One is a text file with only the nucleotide sequence represented by the standard abbreviations, i.e. A,C,T,G, or N when nucleotide has not been identified (see section 1.3). The other file is a electropherogram (or chromatograph) of the same sequence which displays a series of peaks in 4 different colours, each representing a nucleotide (green = **A**; blue = **C**; red = **T**; black = **G**) – see Griffiths *et al.* (1996, p. 448); or Hillis *et al.* (1996c, plate 2) for examples of electropherogram images).

The sequence files can be analysed on the same Machintosh computer linked to the automated sequencer, however, this computer is often only used for the automated sequencing recording. Therefore, files are placed on disk or sent as e-mail attachments, after which can be analysed on other computers. The programs used for sequences analyses in the present work are also Machintosh based, but these files can also be used in other programs that are PC compatible.

The first step to determine the sequence of a particular sample of DNA is to open the sequence file (electropherogram) generated by each primer (tube reaction – see section 9.2.7A) using the **Factura™** program (Factura - Feature identification, version 1.2.Or6 – © Applied Biosystems, Inc.). Immediately, it is possible to tell if the sequence obtained is reliable: the peaks should be clear and high, with very small or basically no background peaks. If the electropherogram shows many overlapping peaks, and many unidentified nucleotide bases (many 'Ns'), the particular sequencing reaction will have to be repeated. The most common cause of a 'bad' sequence is that the excess of terminators (fluorescent labelled dideoxynucleotides) where not effectively removed during ethanol precipitation – see section 9.2.7B. A

short region at the start of all electropherograms normally shows overlapping peaks (area of primer annealing). This area should be cut out from the text file. The last part of the sequence (normally a row of many 'Ns') is also cut out. Sometimes, the sequence is fairly good for a certain number of bases, but from a certain point becomes unreliable; this part should also be cut out, with the remaining data still useful to confirm and determine the sequence. After all the necessary cuts are done, the sequence is saved; in the case of reverse primers, the sequence is saved as its *reverse complement* (do not forget to do this, otherwise you will be unable to align the sequence produced by the different primers run in the same DNA sample).

The program **Sequence Navigator™** (Version 1.0.1, ABI Prism Perkin Elmer – © Applied Biosystems, Inc.) is used to align the sequences of the different primers. A new layout is open, and the sequences, obtained from each of the 4 primers run for a single DNA sample, are imported. Then, the sequences in the layout are selected and aligned using "Clustal" alignment (gap and match penalties = 10). Ideally, the sequences produced by the primers 'ITS 5P & 4' (the latter is the reverse primer) should approximately match each other in size, primer 'ITS 2G' should align in the first half of the sequence (spacer ITS1), and the primer 'ITS 3P' should align in the second half of the sequence (spacer ITS2). To delimit ITS1 and ITS2, it is helpful to locate the 'internal primers', i.e 'ITS 2G & 3P' (see primer sequences in section 9.2.7A) that are within the 5.8S subunit in the middle of the ITS region (see Fig. 9.2). This is especially important if the sequences obtained for the primers were shorter than ideal, so locating the primers shows how much of the sequence we have got. Differences between the sequences of the primers can be highlighted by selecting "create shadows". To check each of the discrepant bases, we go back to the Factura program, open the sequence and locate the particular dubious base in the electropherogram.

In case we are unable to identify one or more bases in the sequences, we can name them using the following symbols: **K** (G or T), **M** (A or C), **R** (A or G), **S** (G or C), **W** (A or T), **Y** (C or T), or **N** (A,C,T, or G) – see also section 1.3. After all the dubious bases have been checked and the sequence of the whole ITS region determined (we may need to repeat the sequencing reactions for some primers), we can copy and save it in a simple text file.

9.2.8 Sequence alignment

After sequences have been determined for all the samples, it is necessary to align them before using the programs of phylogenetic analysis.

Sequence alignment is arguably the stage of phylogenetic analysis requiring the most care. ITS sequences of closely related species are generally easily aligned, even by eye (if not, the putative close relationship is questionable – see discussion on *concerted evolution* in section 9.1.1B - DNA sequencing). However, alignment of many sequences, especially if more distantly related taxa are included, dictates a program for automated alignment, which also avoids preconceptions on relationships.

The main assumptions of sequence alignment are: **1)** The sequences under analysis are **homologous**, i.e. they have a common descent (for discussion of homology of ITS region, see section 9.1.1B - DNA sequencing). **2)** The differences between homologous sequences are the result of: **a)** *nucleotide substitutions*; and, **b)** *insertions* or/and *deletions*, which are generally termed **indels** (often it is not possible to know if a particular 'gap' in the alignment is the result of a deletion in that sequence or an insertion in another).

The general approach in alignment is to use *parsimony*, which favours the fewest changes. Alignment starts as a two sequence comparison ('pairwise alignment'), and is generalised to multiple sequences by the building of a phylogenetic 'tree' (Hein, 1998). As Hein (1998) remarked: "prealigned data [have] been manipulated in a way that could seriously bias the [final] data" for analysis. Therefore, is not only the programs that are used after sequence alignment that affect phylogenetic analysis, but also the program used for multiple alignment.

The program **Clustal X**, version 1.8 (Thompson *et al.*, 1997), was used for multiple sequence alignment [Clustal V and Clustal W are earlier versions of the same program] – Clustal X was used on a Macintosh PowerMac, but is also available for PCs. The program **SeqPup** (version 0.6f, 'child of SeqApp' – a biosequence editor and analysis application – © 1995 D.G. Gilbert) was also indispensable for the final editing on the alignment, for storage of multiple sequence files, and also for the flexibility of export formats available (GenBank, Paup/Nexus, etc).

Multiple sequence alignment was carried out as follows:

All sequences previously stored in simple text files (Macintosh) were copied (one by one) to a SeqPup file, and was saved in GenBank format (this is the most stable format). To carry out multiple alignment, the same SeqPup file was also saved in NBRF format. The NBRF sequence file was opened in the Clustal X program and complete alignment carried out. After alignment, the file was saved and opened in SeqPup, where further manual adjustments of the alignment were carried out. The finally aligned sequences were stored as a SeqPup file in GenBank format.

A region at the start of ITS2 (see Fig. 9.3) was excluded from further analysis as unambiguous alignment could not be obtained. The same region of difficult alignment has been found in the sequences of ITS2 of other plant groups (e.g. in the *Gesneriaceae*). The high variability of this region is related with the secondary structure of the RNA transcript of ITS2, where part of the 1st arm ('loop') appears to be superfluous, and is therefore subjected to a higher rate of mutation, not only in sequence but also in length – J. Denduangboripant, Institute of Cell & Molecular Biology (ICMB), Edinburgh University (personal communication).

9.2.9 Phylogenetic analysis

Final phylogenetic analysis included 61 ITS sequences (see Fig. 9.3), 56 of which were from *Bupleurum* (**ingroup**). The outgroup was selected from other *Apioideae* genera that have appeared more closely related to *Bupleurum* in previous molecular analysis – see Plunkett *et al.* (1996b), Downie *et al.* (1998), Katz-Downie *et al.* (1998). The following 5 taxa were used as an **outgroup**:

- *Anginon difforme* (L.) B.L.Burtt and *A. paniculatum* (Thunb.) B.L.Burtt – ITS sequences obtained in the present work (see Table 9.1).

- *Heteromorpha arborescens* (Thunb.) Cham. & Schltdl. – ITS sequence published by Downie & Katz-Downie (1996); a copy was obtained from the internet at GenBank [http://www3.ncbi.nlm.nih.gov/ – NCBI, National Center for Biotechnology Information, USA].

- *Physospermum cornubiense* (L.) DC. and *Pleurospermum foetens* Franch. – these ITS sequences were kindly provided by Dr Stephen Downie (Department of

Plant Biology, University of Illinois at Urbana-Champaign, USA). For full accession information see, for the first species, Downie *et al.* (1998), and for the second, Katz-Downie *et al.* (1999). These sequences are now also available at GenBank.

There are several important concepts in **cladistics** that should be revised for comprehension of the following discussion on phylogenetic analysis – see e.g. Forey *et al.* (1992), Kitching *et al.* (1998) and Wiley *et al.* (1991). See also Swofford *et al.* (1996), for ample discussion on these methods, including the mathematical reasoning and explanation of algorithms used in computer programs of analysis.

Sequence phylogenetic analysis was carried out using the computer program **PAUP** (*Phylogenetic Analysis Using Parsimony*), which latest version 4.0 (Swofford, 1998) includes options other than parsimony, such as maximum likelihood and distance methods of analysis (neighbour joining and UPGMA) – see section 9.1.2.

The optimality criterion chosen for the main analysis in the present work was **maximum parsimony**. Parsimony favours the shortest evolutionary pathway (= shortest *tree topology* or *cladogram*) and assumes that shared character-states derive from common descent, i.e. they are homologous (here *homology* equals *synapomorphy* – see Patterson, 1982). However, conflicts between the shortest tree topology and the distribution of character-states may occur, with some appearing to have evolved independently in different branches (*clades*) of the tree. In this situation, the character-states do not represent a synapomorphy, and are therefore not homologous; **homoplasy** is then said to be present. Homoplasies originate either by evolutionary *convergence*, *parallelism* or *reversal* of a character to a plesiomorphic (ancestral) state. The latter is particularly common in DNA sequences, as there are only 4 possible states (A,C,T,G), with *transitions* (C $\leftrightarrow$ T or A $\leftrightarrow$ G) being more common than *transversions* (purine to pyrimidine or *vice-versa*).

In parsimony analysis there are 3 options when looking for the shortest tree: **a)** *Exhaustive search*, which evaluates all possible trees. **b)** *Branch-and-bound search*, which uses an algorithm that discharges trees without evaluating them if they meet certain criteria, and so it considerably reduces the number of trees examined. **c)** *Heuristic search*, which only evaluates a small portion of all possible trees. The first

two procedures are exact methods, i.e. they will provide the shortest tree for the data. However, with the increase of the number of taxa under analysis (more than 16-20 taxa – depending on size of the data matrix), the number of possible trees becomes astronomical, and **heuristic search** will have to be chosen. The shortest tree(s) found by this procedure may not be 'optimal', as the search is constrained by the way the taxa are added. In order to optimize heuristic search, it is recommended to carry out **branch swapping**, which involves moving branches to different parts of the tree, and then further evaluating these new tree topologies; increasing the amount of branch swapping greatly improves the chances of getting the 'optimal' (= shortest of all possible) trees.

There are two algorithms to optimize character-state distribution in the trees: **a)** ACCTRAN (*accelerated transformation*) "accelerates the evolutionary transformation of a character", i.e. it places character changes on the tree as close to the root as possible, and so homoplasies will be accounted as reversals to the plesiomorphic state. **b)** DELTRAN (*delayed transformation*) "delays the transformation of a character", i.e. it places character changes as far as possible from the root of the tree, favouring parallelisms over reversals when chance is given. These algorithms evaluate the most parsimonuous trees (equal length) that have been found, and select those that better fit a particular sort of data. For additional explanation on tree building and optimization, see e.g. Wiley *et al.* (1991, p. 45-69). ACCTRAN is generally the choice when analysing DNA sequences (it is set as *default* in PAUP program), and, as said above, reversals are not uncommon events in the evolution of DNA.

The following **strategy** was used for analysis in **PAUP 4.0** (version 4.0b2) – this is a '*beta version*' and the software is still under test; but the checking has been going for considerable time and no serious problems have been detected [major changes were introduced in the previous version of PAUP (3.1.1), e.g. the inclusion of distance and maximum likelihood methods].

1) Create a Paup file – This is easily done by saving the aligned SeqPup file in GenBank format (see previous section - 9.2.8) as a new file in Nexus format; when opening this file within the PAUP program, a Paup file is automatically created.

2) 'Execute' the Paup file (if not already done when initially opening the file) – see menu under 'file'.

3) Select program settings for analysis:

a) Settings used for parsimony analysis:

- *General search options*: collapse branches if maximum length is zero.
- *Character-state optimization*: ACCTRAN.
- Multistate data (i.e. a single taxon shows multiple states for a single character) is interpreted as uncertainty – cases of not identified bases (N), or when they were recorded as W (A or T), Y (C or T), etc.
- Gaps (i.e. *indels*) treated as missing data (i.e. they are not coded as special new characters, like particular deletions).

b) Options selected for heuristic search:

- *General search options*: best trees only.
- *Starting-tree options*: a) get by stepwise addition; b) swap on best trees only when multiple starting trees exist.
- *Stepwise-addition options*: addition sequence - random; 1000 replicates; hold one tree at each step.
- *Branch-Swapping options*: Tree Bisection-Reconnection (TBR) [this is the option that performs more branch-swapping in the trees], saving no more than 20 trees per replicate.

c) Other options (set as *default*) in PAUP:

- Characters were unordered and equally weighted.
- Parsimony-uninformative characters (i.e. *autapomorphies*) were included in the analysis (contributing therefore to total length of the trees).

4) After *rooting* (i.e. selecting the outgroup taxa) carry out heuristic search.

5) At the end of the analysis, save the shortest tree(s) found to a file. If more than one equally parsimonous tree has been found, the *strict consensus tree* (the tree that only shows the monophyletic groups present in all the competing shortest trees) can then be produced.

To estimate clade support, two **randomization procedures** were chosen – for detailed explanation see Kitching *et al.* (1998, p. 119-138), and Li (1997, p.141-146):

Bootstrap:

This computer procedure randomly samples the characters of the data matrix creating a 'pseudoreplicate' data set of similar size than the original (i.e. with the same number of characters). Then, phylogenetic analysis (heuristic search in the

present case) is carried out. This is repeated the number of times we desire (number of *replicates*). At the end, a *consensus tree* of all the shortest trees found is produced, and each branch on the tree (except if terminal) will have a numerical value that represents the percentage of the trees that show that particular clade. Clades that appear in less than 50 % of the trees will not be shown. Bootstrap values between 50-74 % are considered low; 75-84 % moderate; and 85-100 % represents strong support.

In the present work, bootstrap was carried out in PAUP as 'full heuristic search' with the same options chosen as indicated above (1000 replicates, TBR branch-swapping algorithm, saving 20 trees per replicate), with the exception that 'simple' sequence addition for creating the replicates was selected (instead of random).

Jackknife:

This is a similar procedure to bootstrap, the difference is that the random sampling of characters is carried out without replacement, i.e. characters are randomly removed from the data matrix, and the final replicates are smaller than the original. The normal option is to do jackknifing with 50 % character deletion. A Jackknife consensus tree is produced in the same way than bootstrap, with percentages values of the clades showing their degree of confidence. Jackknife was carried out in PAUP as "fast-stepwise" addition of 10,000 replicates.

Distance Analysis:

The latest version of PAUP includes distance methods of analysis (Neighbour Joining and UPGMA). **Neighbour joining** is a clustering method, with very fast analysis that generally produces a single tree. It is a good method for estimating the *minimum evolution* tree, but it suffers from the limitation that is not an 'optimality method' (Page & Holmes, 1998, p. 184) – i.e. it may not find the shortest tree, or other tree options of similar length that may be available are not shown.

In order to compare the results of cladistic and distance analysis, neighbour joining analysis was also performed for the data matrix of the aligned ITS sequences. The 'default' distance settings of PAUP were used.

9.3 Results

9.3.1 Sequence analysis

A total of 68 samples were sequenced for ITS region: 66 from *Bupleurum*, representing 32 species (35 taxa), and two other samples from *Anginon* (two species) – see Table 9.1. In total ITS was sequenced for the first time for 33 species: 31 from *Bupleurum* (*B. falcatum* had been already sequenced – Lee & Rasmussen, 1998; Valiejo-Roman *et al.*, 1998), and the two species from *Anginon*.

Of the 66 sequences of *Bupleurum*, only 61 were used in the final analysis (see Fig. 9.3 for final alignment of sequences). Some of the sequences obtained for the same taxon/population were identical, and only one for each was used (see Table 9.2). This happened for *Bupleurum fruticosum* (Acc. No 243 = 248), *B. gibraltarium* (Acc. No 245 = 248), and in the two distinct populations of *B. acutifolium*: Spain (Acc. Nos 262 = 263 = 305) and Portugal (Acc. No 228 = 240). Some other samples for a single species showed little variation (1-3 bp; sometimes including unidentified nucleotides), but they were all used in the analysis (e.g. the 3 samples of *B. montanum*).

Also, identical ITS sequences were obtained for different taxa, which have been generally regarded as different species (*Bupleurum fruticescens* and *B. spinosum*), but in this case I was already considering these as single species (see section 9.4 and *B. fruticescens* in chapter 10). The sequences obtained for *Bupleurum fruticescens* subsp. *fruticescens* (taxon only present in Spain - Acc. No 238 = 253) and subsp. *spinosum* (Spanish samples: Acc. Nos 249 = 259) were all identical – NW African samples of '*B. spinosum*' showed slightly different sequences. As '*Bupleurum spinosum*' has generally been considered a different species, a sequence for each of the Spanish taxa of what I regard as *B. fruticescens* (one for subsp. *fruticescens* and one for subsp. *spinosum*) was included in the analysis, despite knowing that they were identical.

In the case of NW African endemic taxa, very little variation, if any was found between some of the species, such as *B. benoistii*, *B. lateriflorum*,

B. plantagineum and *B. montanum* – see Figs 9.5 and 9.6, where length was only of a few steps or even zero for some of the taxa (see section 9.4 for discussion).

Of the clearly delimited species in the area under study (W Mediterranean and Macaronesia as defined in chapter 4), there is only one species missing in the ITS analysis: *Bupleurum foliosum*. Several attempts were made to extract DNA from samples of *B. foliosum* (Acc. No 275 & 278), and, even when finally a slight band of total DNA was obtained (using DNeasy plant mini kit), PCR amplification totally failed (alternative primers 'ITS 1' and 'ITS 8' were also used – see section 9.2.5).

Several attempts were also made to amplify the ITS region from DNA extracts of *Glia prolifera* (Acc. No 284) with no success – *Glia*, from S Africa, is another of the possible allied genera of *Bupleurum*; so the intention was to use it as an outgroup.

Character information:

- Total number of characters analysed: **615** (an area of 60 bases of ambiguous alignment near the start of ITS2 was not included in the analysis) – see Fig. 9.3.

- **322** characters are constant – note that 5.8S subunit (164 bp long), a conservative gene, was included in the analysis; also included were 15 bp of the end of the 18S subunit and another 15 bp of the start of the 26S subunit (the latter shows however one varying character).

- **235** characters are parsimony-informative (= **synapomorphies**).

- **58** variable characters are parsimony-uninformative (= **autapomorphies**). [an autapomorphy is a derived character that is restricted to a single terminal taxon].

The following table summarizes length variation of the different genes of the ITS region:

Sequence	Length range (bp)	Aligned length (bp)
ITS1	173-218	230
5.8S	164	164
ITS2	213-230	251
ITS region	567-610	645

9.3.2 Phylogenetic analysis

Heuristic search of the aligned sequences (Fig. 9.3) produced 128 maximally equally parsimonous trees, all of which were 765 steps long. Consistency and retention indices are indicated in the legend and were the same for all the trees.

The '*overall*' or '*ensemble*' **consistency index (CI)** is a measure of the degree of homoplasy in a particular tree; CI equals to the minimum amount of possible evolutionary change (character changes) divided by the actual number of changes/steps (tree length) of a particular tree [*c* or *ci* is the consistency index shown by the transformation series of a single character]. The highest possible value is CI = 1.0, which means that the tree does not show homoplasies. A low consistency index indicates that many characters contradict the tree topology. However, CI is inflated by parsimony-uninformative characters, such as autapomorphies; so, if there are many of them, it may appear that many characters support the tree. To avoid this problem the ensemble **retention index (RI)** is used; RI measures the amount of similarity that corresponds to actual synapomorphy in a given tree, as it only uses parsimony-informative characters. The **rescaled consistency index (RC)** excludes the characters that inflate the CI; RC is the product of the CI and the RI. For detailed explanation of the various indices see Kitching *et al.* (1998, p. 92-99), Judd *et al.* (1999, p. 21-22), and Wiley *et al.* (1991, p. 72-76).

Figure 9.4 shows the strict consensus tree produced from all the 128 maximally parsimonous trees; it also includes the values of clade support obtained from bootstrap and jackknife analysis (see section 9.4 for further discussion). Figures 9.5 and 9.6 show two of these parsimonous trees, and give an idea of the sort of variation found in the placing of the different taxa along the trees.

Fig. 9.8 shows one of the 16 maximally parsimonous trees obtained from heuristic analysis of the 5.8S subunit alone – the strict consensus tree is not shown as too little variation exists, and the tree is visually not easy to understand.

Fig. 9.7 shows the single tree obtained after neighbour joining analysis. Notice that this tree is in total agreement with the strict consensus tree (Fig. 9.4). It is also very similar to several of the maximally parsimonous found by cladistic analysis.

Table 9.2 - Names of taxa and accession numbers corresponding to the abbreviations used in Fig. 9.3-9.8 for the sequences. For further details on accessions see Table 9.1 (p. 129) and Appendix II. When more than one accession no. is indicated for a single abbreviation, it means that the ITS sequences of these samples are identical.

Sequenced taxa		Abbreviation	Acc. Nos
Anginon difforme		Ang.diffo	193
A. paniculatum		Ang.panic	313
Bupleurum acutifolium	Portugal	B.acut.Po	228, 240
	Spain	B.acut.Sp	262, 263, 305
B. album		B.albu269	269
B. angulosum		B.angu224	224
B. balansae		B.bala268	268
		B.bala302	302
B. baldense		B.bald276	276
B. barceloi		B.barc295	295
B. benoistii		B.beno285	285, 309
		B.beno300	300
B. canescens		B.cane301	301
B. dumosum		B.dumo293	293
B. falcatum		B.falc282	282
B. fruticescens subsp. *fruticescens*		B.frutice	238, 253
B. fruticosum		B.frutico	243, 248
B. gerardii		B.gera17a	17a
		B.gerar67	67
		B.gera306	306
		B.gera307	307
B. gibraltarium		B.gibr245	245, 252
B. canescens 'var. *handiense*' [= *B. handiense*]		B.handi28	28
		B.hand207	207
B. lancifolium		B.lanc287	287
B. lateriflorum		B.late279	279
		B.late303	303
B. longifolium		B.long310	310
B. montanum		B.mont264	264
		B.mont292	292

Table 9.2 - Continuation from previous page.

Sequenced taxa		Abbreviation	Acc. Nos
Bupleurum montanum		B.mont304	304
B. mundii		B.mund283	283
B. odontites		B.odon291	291
B. oligactis		B.olig265	265, 298
[= *B. atlanticum*]		B.olig281	281
B. plantagineum		B.plant272	272
B. praealtum		B.prae267	267
		B.prae288	288
		B.prae289	289
		B.prae308	308
B. ranunculoides		B.ranu181	43, 181
		B.ranu296	296
		B.ranu297	297
B. rigidum subsp. *paniculatum*		B.rigpa70	70
		Brigpa244	244
B. rigidum subsp. *rigidum*		Brigrl254	254
		Brigri261	261
B. rotundifolium		B.rotund4	4
B. salicifolium	Canary Islands	B.salic29	29
		B.sali294	294
	Madeira	B.sali.Ma	273
B. semicompositum		B.semi286	286
B. fruticescens subsp. *spinosum*		B.spin.Sp	249, 259
		B.spin280	280
		B.spin311	311
B. stellatum		B.stel312	312
B. subspinosum		B.subs299	299
B. tenuissimum		B.tenu233	233
Heteromorpha arborescens		Het.arbor	See Downie & Katz-Downie (1996)
Physospermum cornubiense		Physo.cor	See Downie & al. (1998)
Pleurospermum foetens		Pleur.foe	See Katz-Downie & al. (1999)

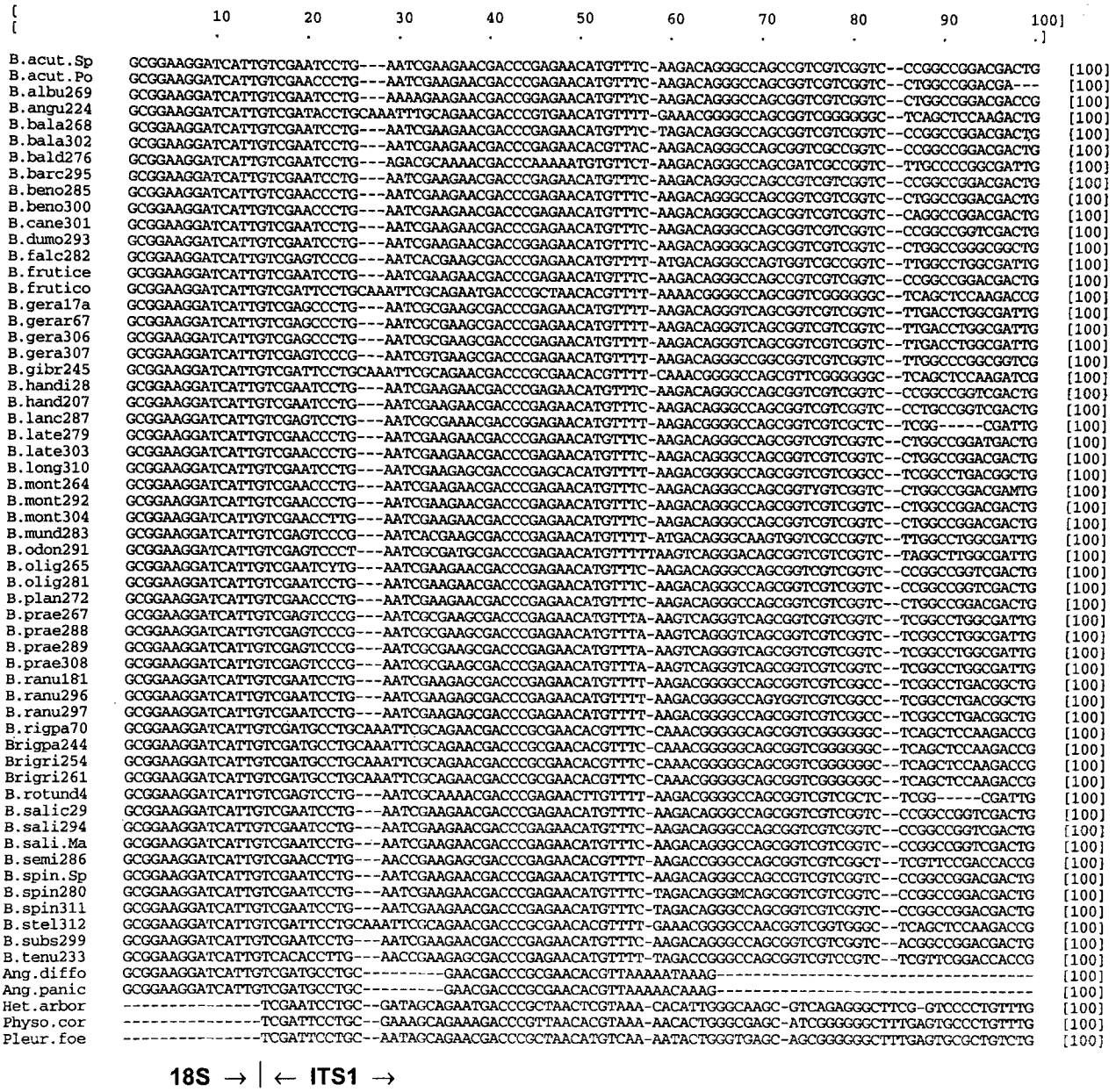


Fig. 9.3 - Aligned DNA sequences of the ITS region in the nuclear ribosomal DNA (nrDNA) from *Bupleurum* taxa. Sequences of 4 other *Apioideae* genera (*Anginon*, *Heteromorpha*, *Physospermum* and *Pleurospermum*) were included as outgroup in the phylogenetic analysis. ITS sequences from *Heteromorpha*, *Physospermum* and *Pleurospermum* were obtained respectively by Downie & Katz-Downie (1996), Downie *et al.* (1998) and Katz-Downie *et al.* (1999) – 5.8S sequences were not available for these taxa, hence the gap in this area. Complete names of taxa are provided in Table 9.2. Nucleotide sites are numbered 5' to 3' from the end of the 18S subunit to the start of the 26S subunit of nrDNA. Hyphens indicate gaps (*indels*) required for alignment. Asterisks below the sequences indicate a region of 60 bp (start of ITS2) excluded from analysis as unambiguous alignment could not be obtained. See section 1.3 for explanation of nucleotide abbreviations.

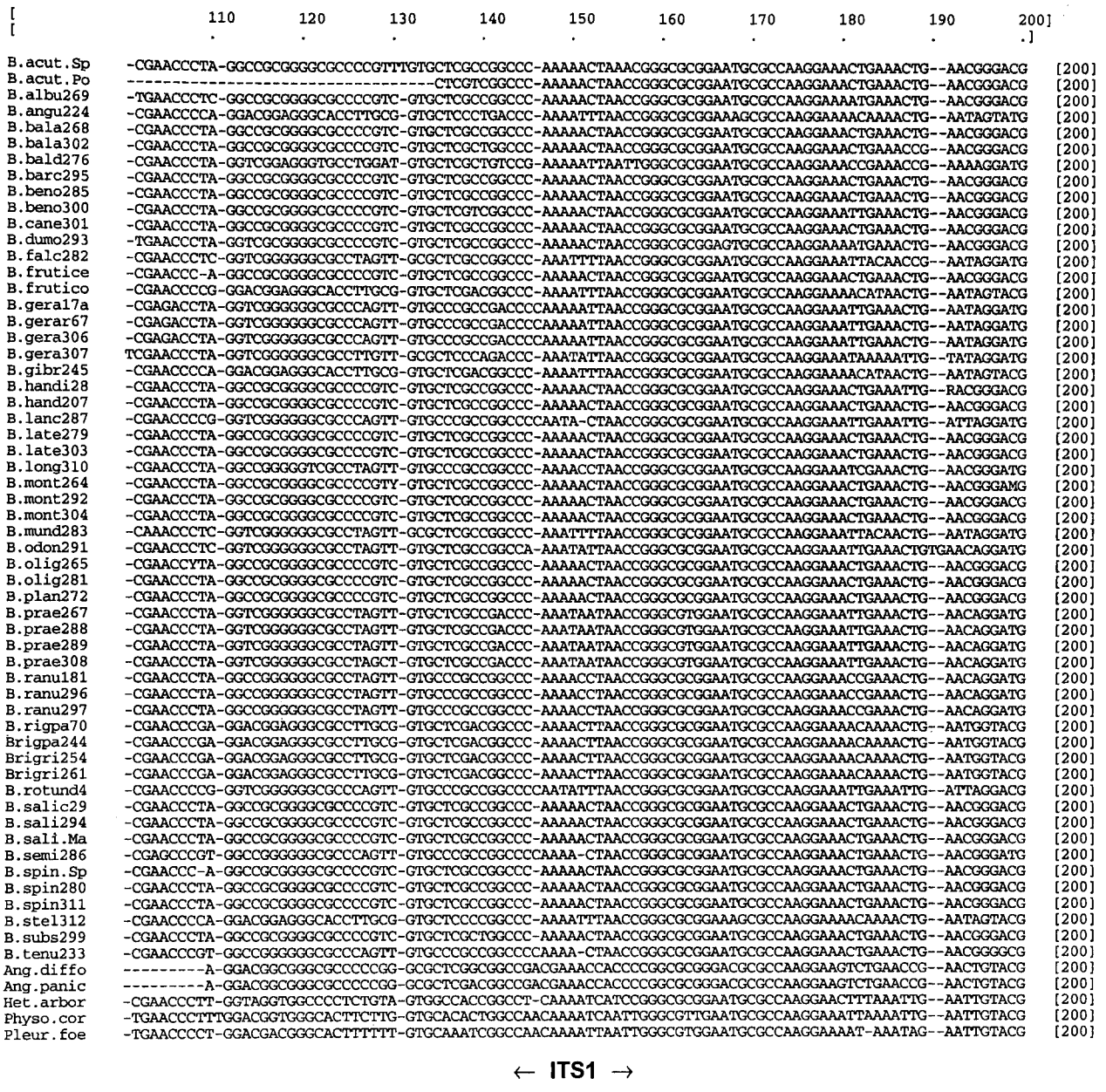


Fig. 9.3 - Continuation from previous page.

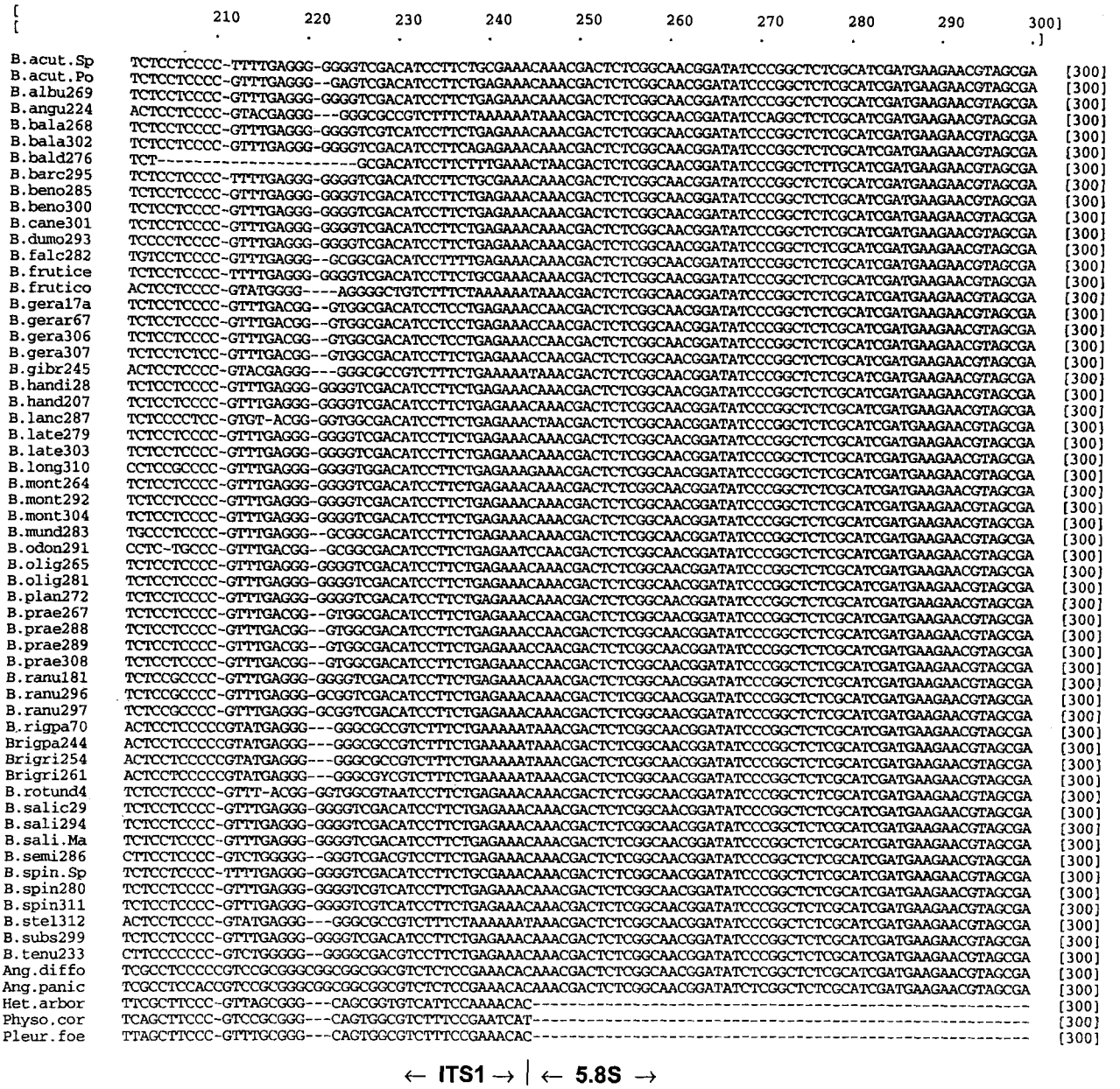


Fig. 9.3 - Continuation from previous page.

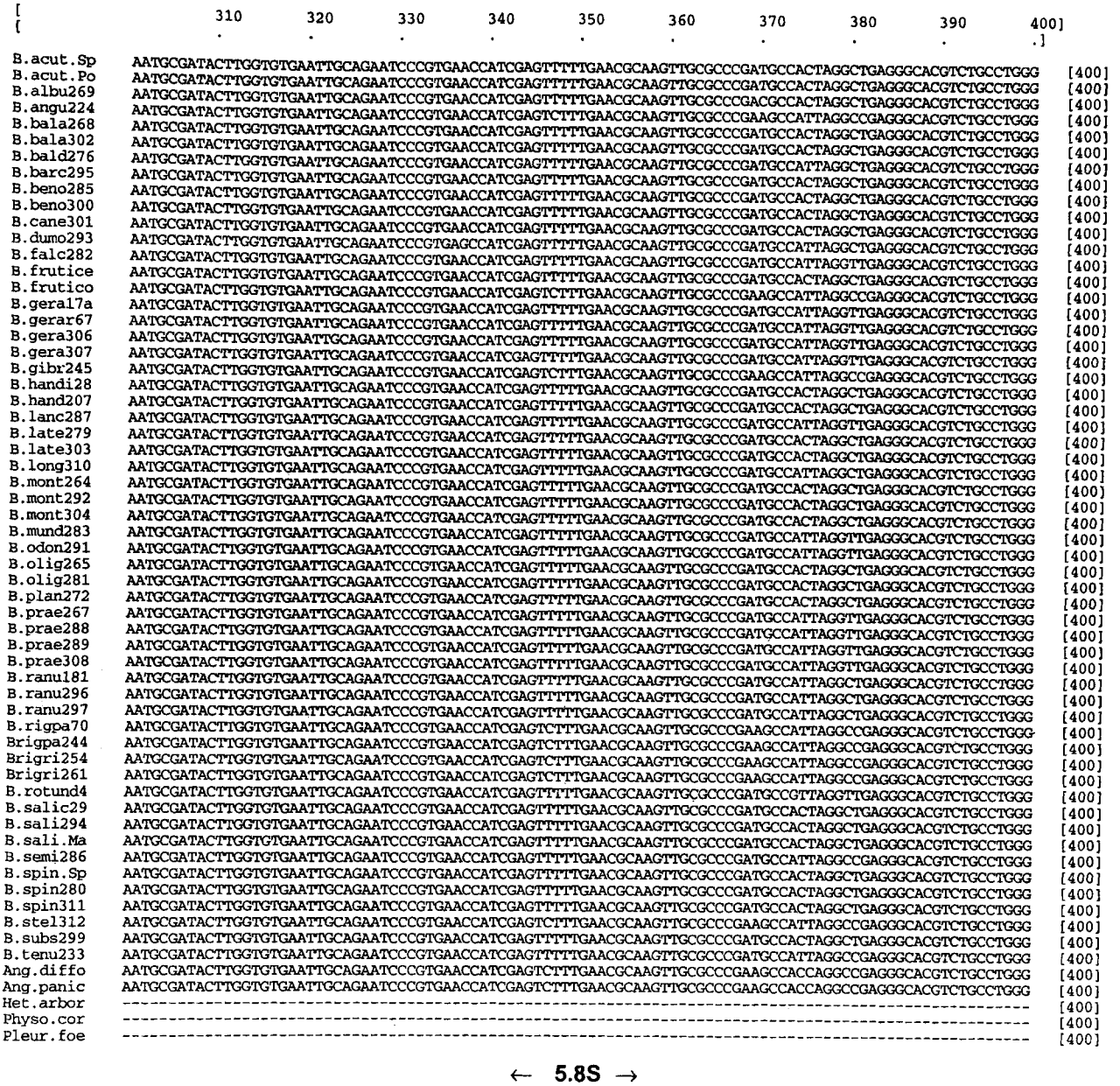


Fig. 9.3 - Continuation from previous page.

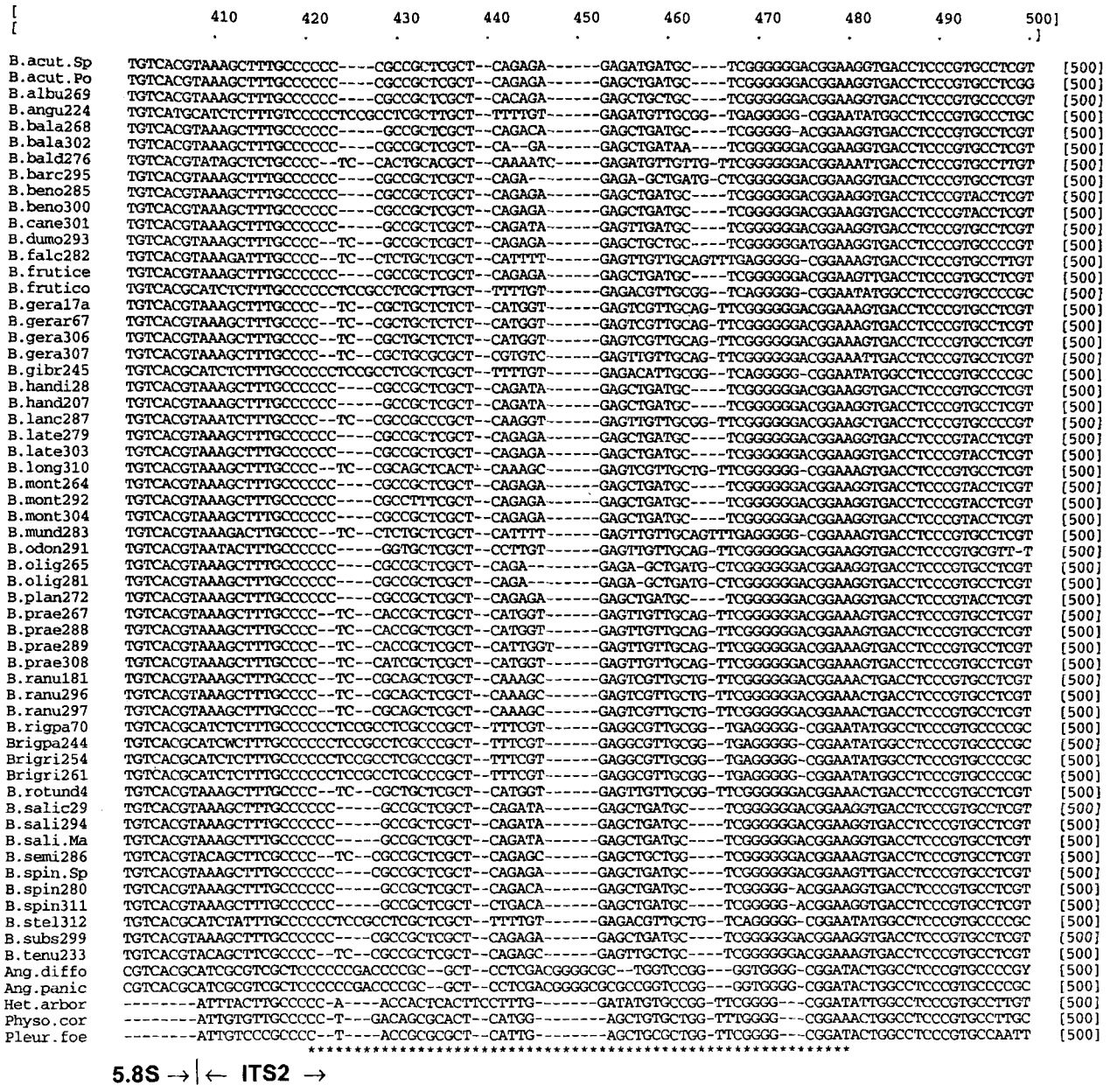


Fig. 9.3 - Continuation from previous page.
(asterisks below the sequences indicate an area of ambiguous alignment excluded from analysis).

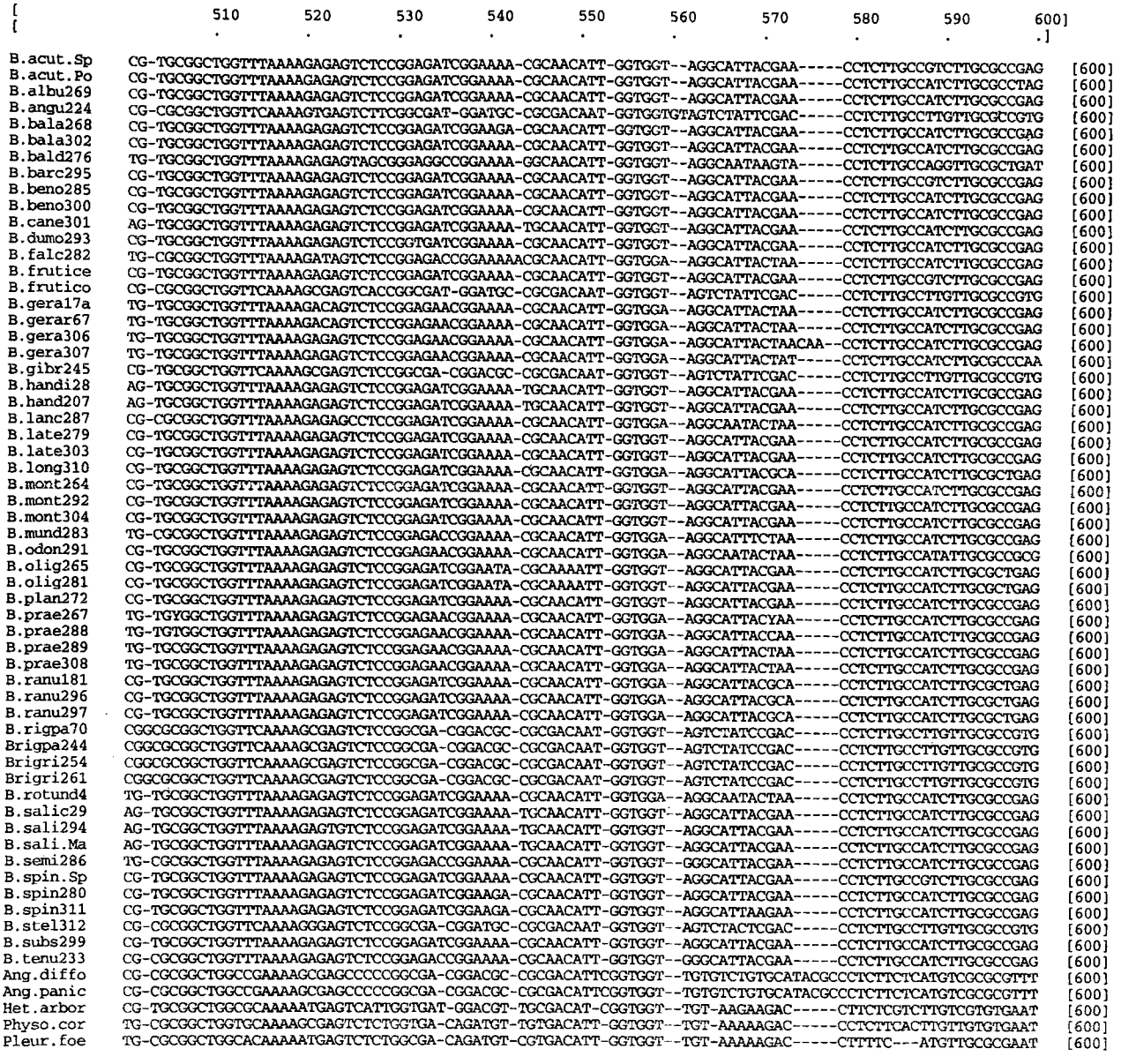


Fig. 9.3 - Continuation from previous page.

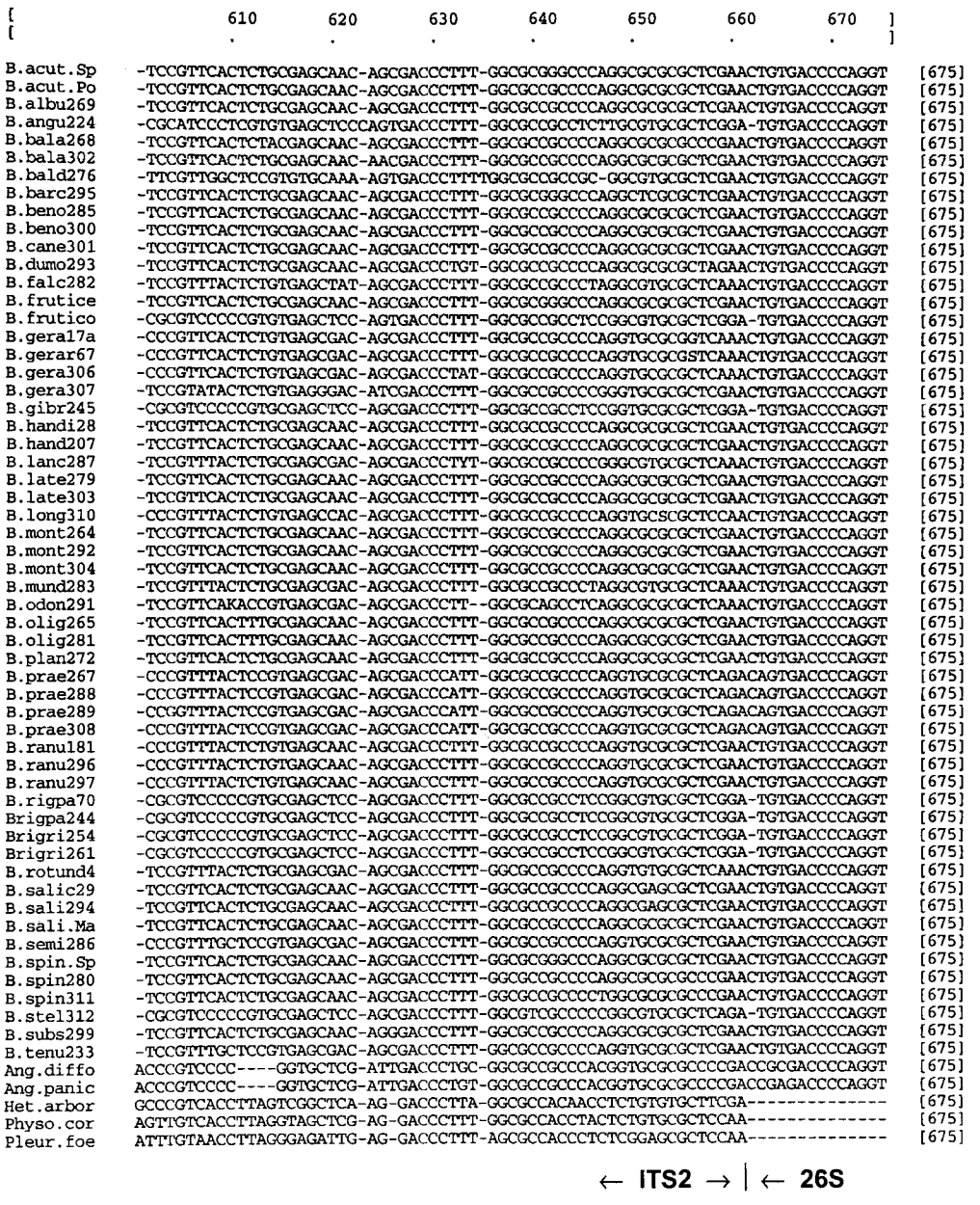


Fig. 9.3 - Continuation from previous page.

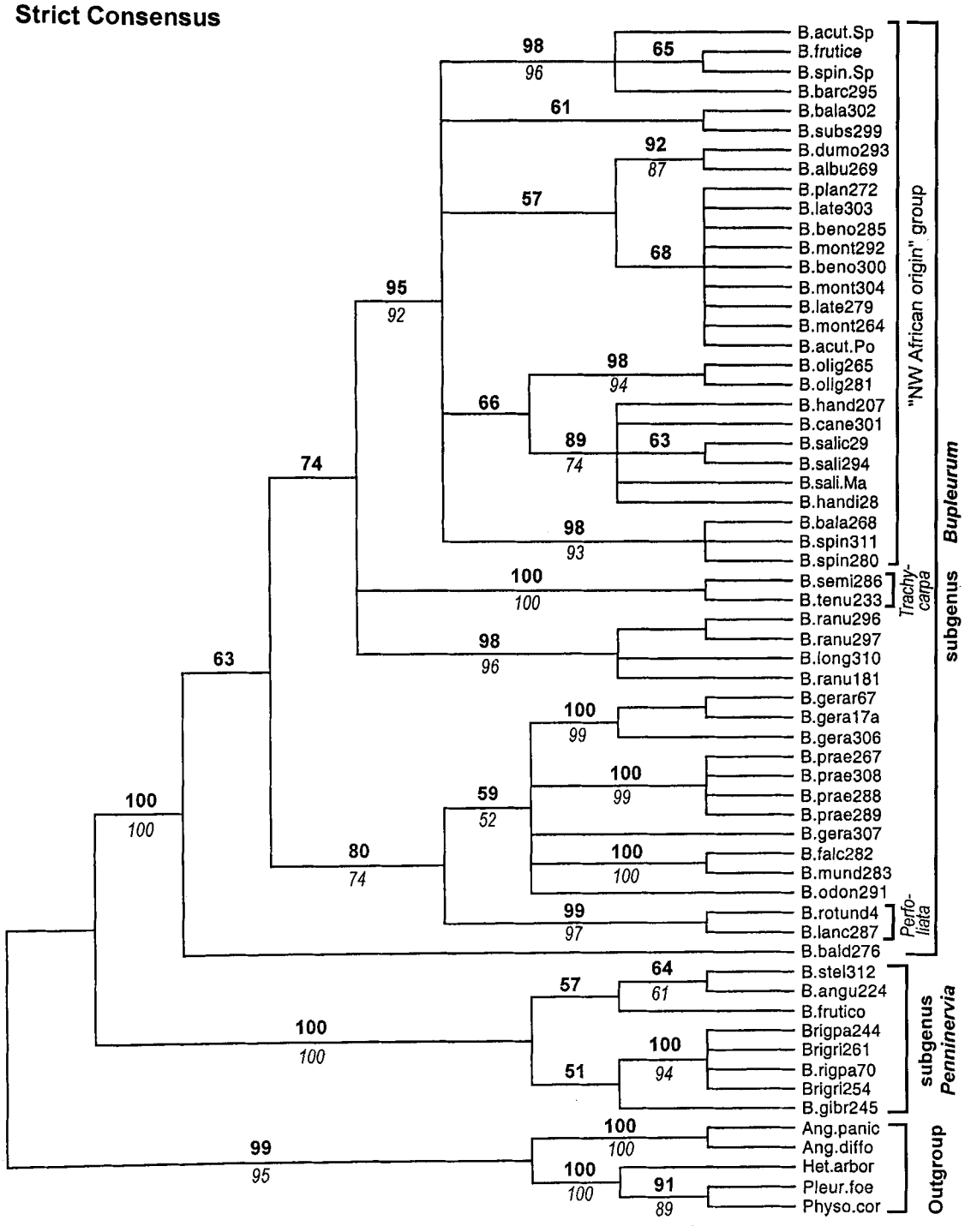


Fig. 9.4 - Strict consensus tree of 128 maximally parsimonious trees based on sequences of ITS region of *Bupleurum*. Outgroup: *Anginon*, *Heteromorpha*, *Physospermum* and *Pleurospermum*. Phylogenetic analysis performed using PAUP version 4.0b2 (Swofford, 1998). Bootstrap percentages (1000 replicates) appear above the branches. Jackknife percentages (10000 replicates) are given below the branches. See Fig. 9.3 (p. 168) for alignment of sequences. Complete names of taxa are provided in Table 9.2 (p. 166).

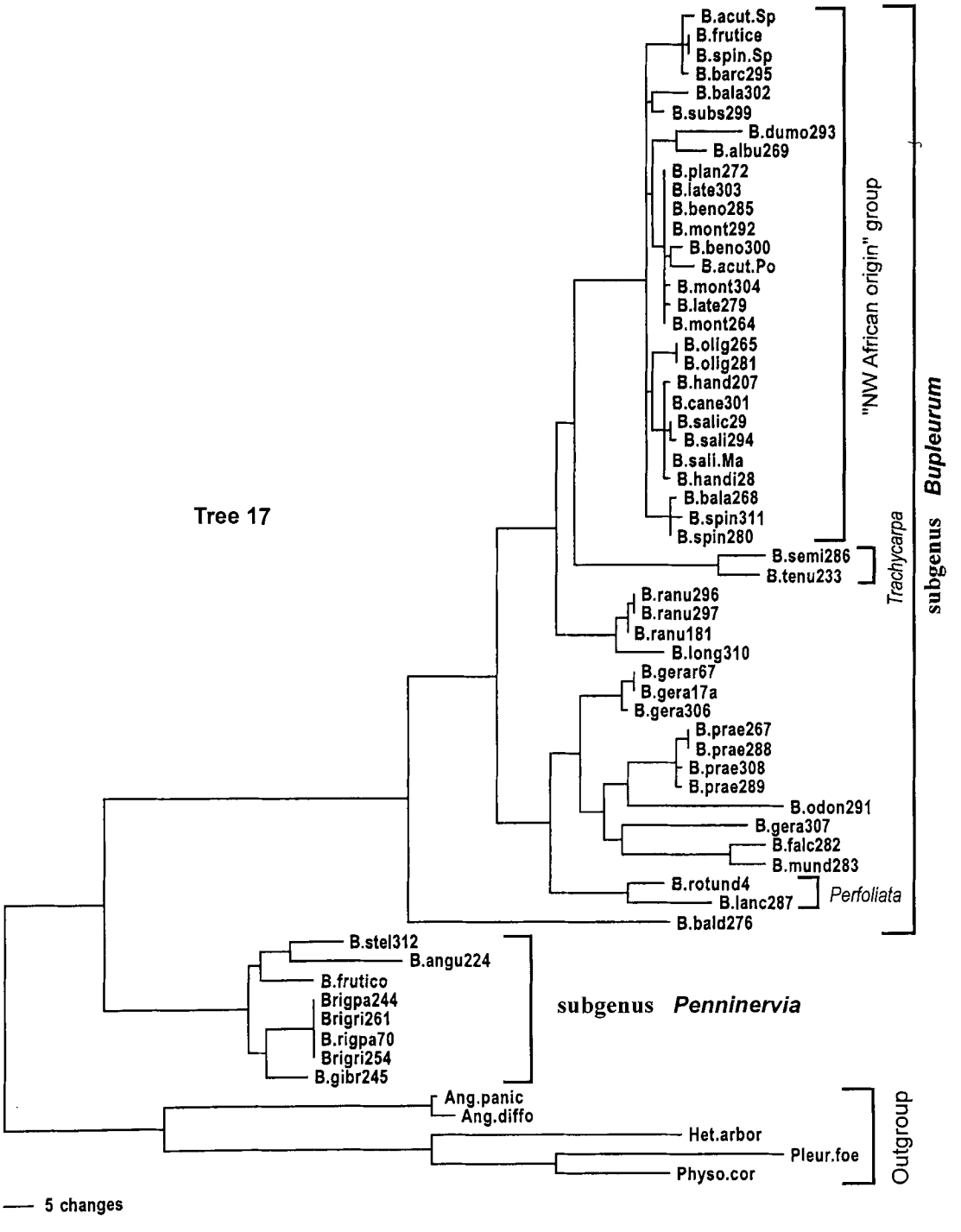


Fig. 9.5 - One of 128 maximally parsimonious trees derived from parsimony analysis of sequences of the ITS region of *Bupleurum*. Outgroup: *Anginon*, *Heteromorpha*, *Physospermum* and *Pleurospermum*. Phylogenetic analysis performed using PAUP version 4.0b2 (Swofford, 1998). Tree length = 765 steps; consistency index (CI) = 0.6209; retention index (RI) = 0.8497; rescaled consistency index (RC) = 0.5276; homoplasy index (HI) = 0.3791. Complete names of taxa are provided in Table 9.2 (p. 166).

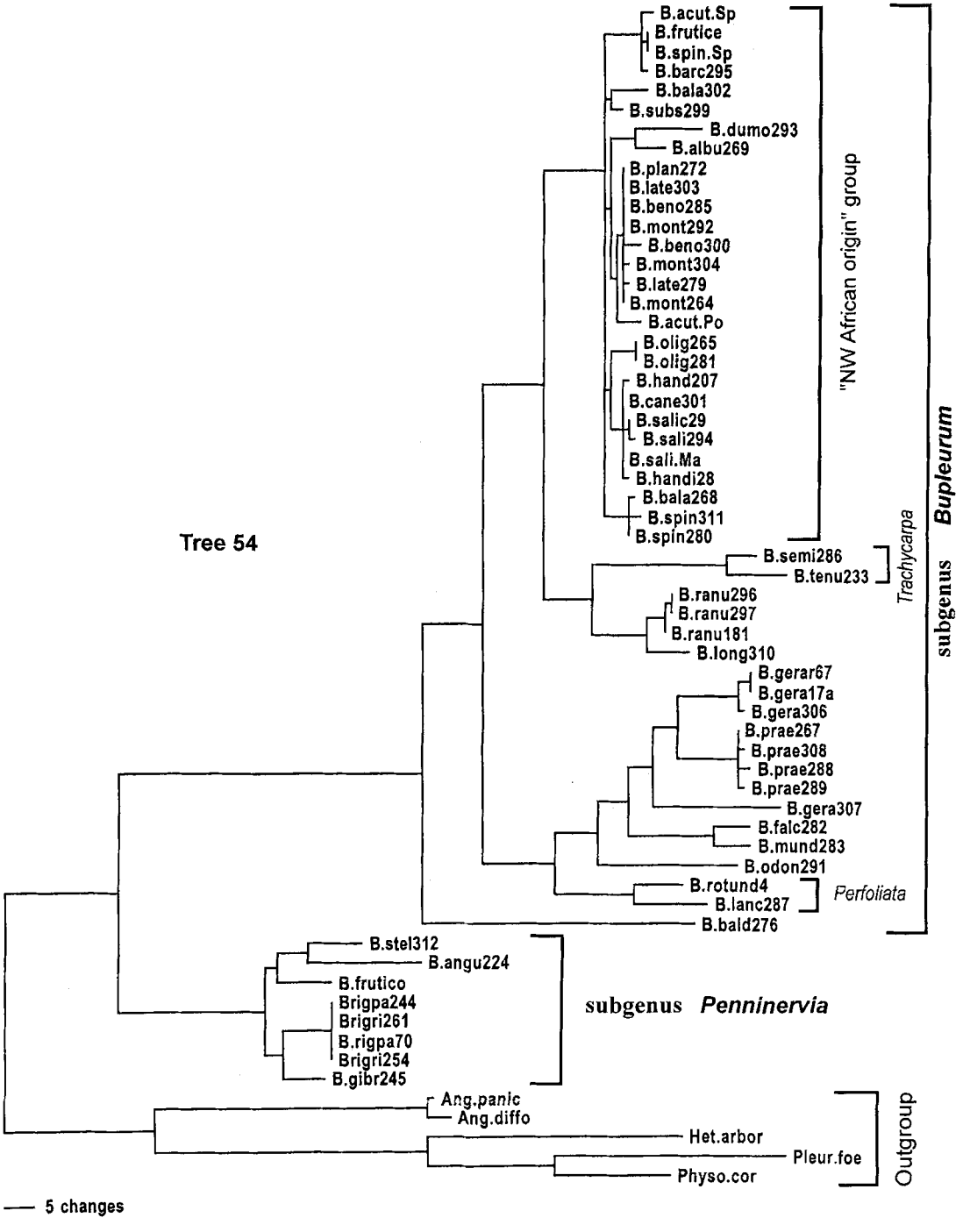


Fig. 9.6 - One of 128 maximally parsimonious trees derived from parsimony analysis of sequences of the ITS region of *Bupleurum*. Outgroup: *Anginon*, *Heteromorpha*, *Physospermum* and *Pleurospermum*. Phylogenetic analysis was performed using PAUP version 4.0b2 (Swofford, 1998). Tree length = 765 steps; consistency index (CI) = 0.6209; retention index (RI) = 0.8497; rescaled consistency index (RC) = 0.5276; homoplasy index (HI) = 0.3791. Complete names of taxa are provided in Table 9.2 (p. 166).

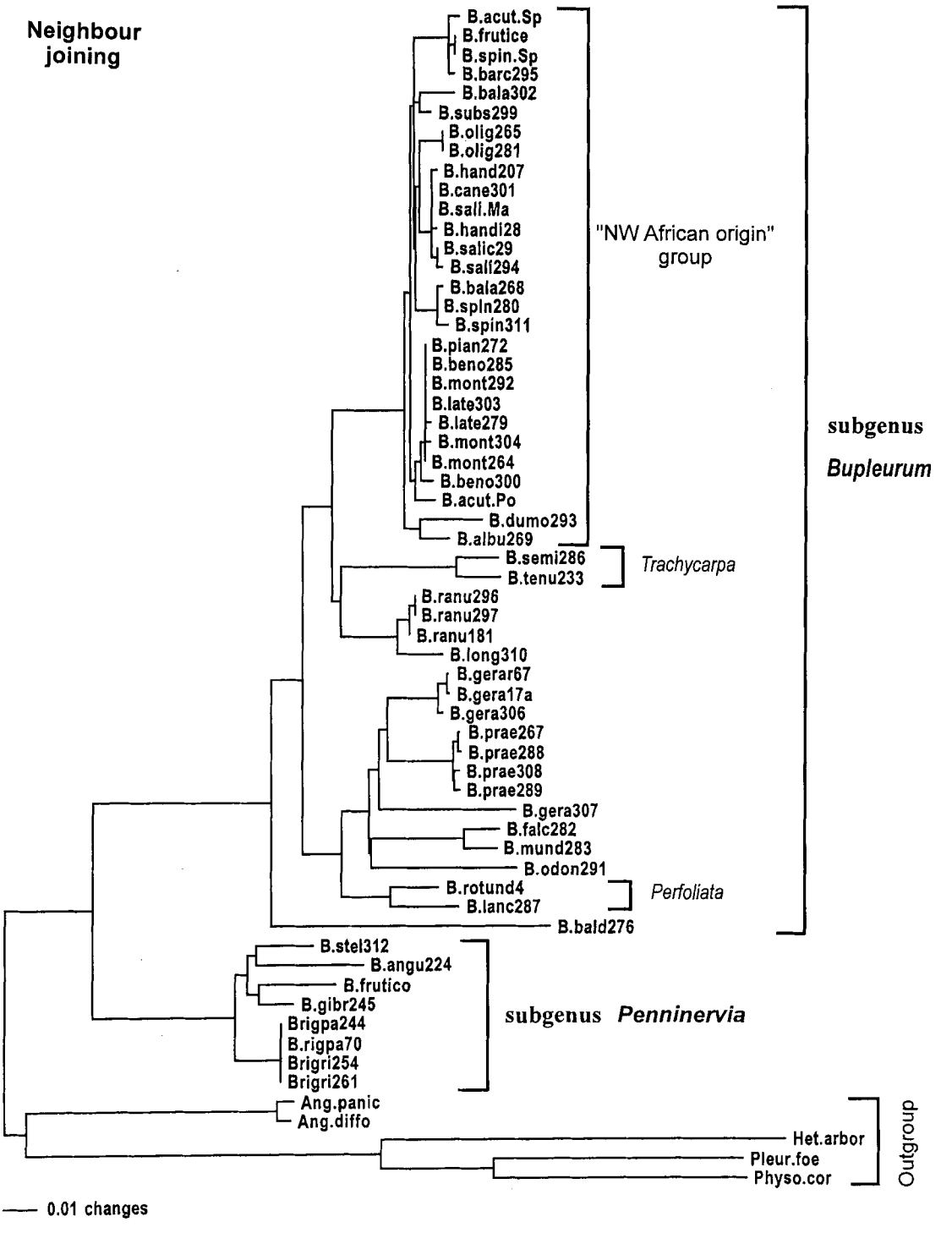


Fig. 9.7 - Single tree obtained from neighbour joining analysis of substitution rates on sequences of the ITS region of *Bupleurum*. Outgroup: *Anginon*, *Heteromorpha*, *Physospermum* and *Pleurospermum*. Analysis performed using PAUP version 4.0b2 (Swofford, 1998). Length of branches are proportional to distances. Complete names of taxa are provided in Table 9.2 (p. 166).

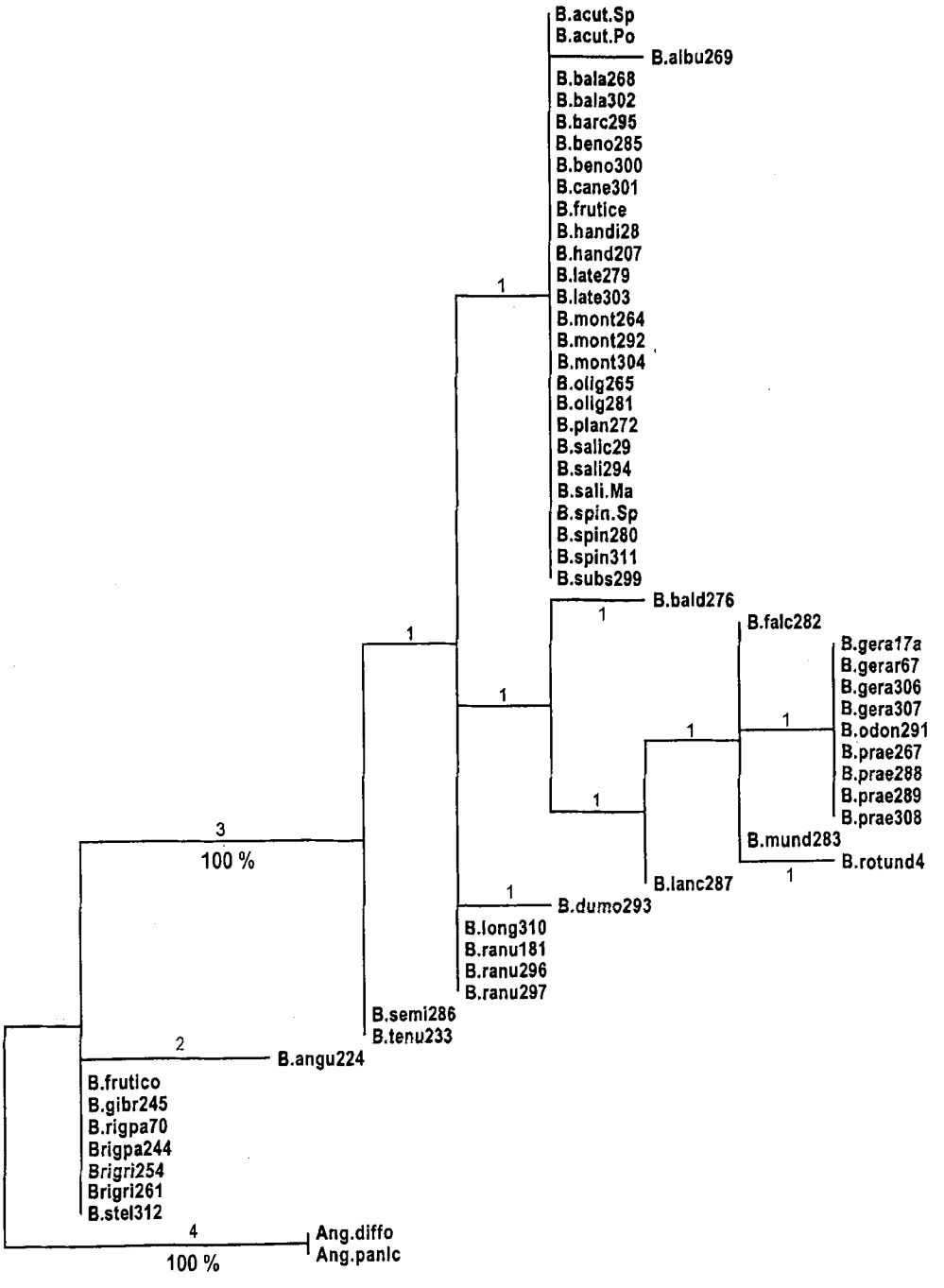


Fig. 9.8 - One of 16 maximally parsimonious trees derived from parsimony analysis of 5.8S sequences of *Bupleurum*. Outgroup: *Anginon*. Analysis performed using PAUP version 4.0b2 (Swofford, 1998). Tree length = 19 steps; consistency index (CI) = 0.895; retention index (RI) = 0.978; rescaled consistency index (RC) = 0.875; homoplasy index (HI) = 0.105. Complete names of taxa are provided in Table 9.2. As expected, the 5.8S subunit, a short coding region (164 bp long), shows only a few (10) parsimony informative characters. Nevertheless, analysis of the 5.8S supports the main dichotomy shown in *Bupleurum* by ITS1 and ITS2.

9.4 Discussion

The phylogenetic analyses of sequences of the ITS region in *Bupleurum* confirms that the genus is a monophyletic group. A very important finding of these analyses is that the genus shows a basal division into two main groups, which are very strongly supported (100 %) in bootstrap and jackknife analyses – see the strict consensus tree in Fig. 9.4, and two examples of maximally parsimonous trees in Figs 9.5 & 9.6 (all the parsimonous trees show the two main clades). Distance analysis (neighbour joining) of the same sequence data also shows the main dichotomy of the genus (see Fig. 9.7).

The 5.8S is a conservative coding region located between ITS1 and ITS2, and has been little used in phylogenetic analysis as it is too short (164 bp) to show enough informative variation for unambiguous analysis. However, it is extremely significant that the few informative characters shown by the 5.8S alone also support the main division of the genus into two major clades. This division is shown by all 16 maximally parsimonous trees produced in the analysis of 5.8S (100% bootstrap support) – Fig. 9.8 shows one of these trees (notice that consistency and retention indices of the trees are very high, indicating low homoplasy).

This finding is specially significant because the taxa studied are a fairly representative sample of the morphological variation found in the genus, as species are included from all the currently accepted sections (see below; and also Tutin, 1968). Therefore, there are implications on the classification of the genus that go beyond the taxa of the region under study (W Mediterranean and Macaronesia). Three of the species sequenced do not belong to this area: *B. longifolium* L. (from C Europe extending to E Russia), *B. mundii* Cham. & Schltdl. (S Africa) and *B. stellatum* L. (European Alps and Corsica). The following are the currently accepted main divisions of the genus *Bupleurum*, with indication of the species sequenced in the present work:

- Section *Bupleurum* ('*Perfoliata*'): *B. lancifolium* and *B. rotundifolium*.
- Section *Coriacea*: *B. fruticosum* and *B. gibraltarium*.
- Section *Diaphyllum*: *B. longifolium*.
- Section *Reticulata* (only two species): *B. angulosum* and *B. stellatum*.

- Section *Isophyllum*: the rest of the species sequenced – see *Bupleurum* species in Tables 9.1 (p. 129) & 9.2 (p. 166).

Although two of these sections are likely to correspond to monophyletic groups (Sect. *Bupleurum* and Sect. *Reticulata*). This sectional division of the genus is not supported by phylogenetic analysis of the ITS region.

The most recent infrageneric classification of *Bupleurum* was proposed by Cauwet (1976), in her PhD thesis on the genus (see Taxonomic History in chapter 2). Cauwet based her considerably modified classification (see Table 2.1, p. 22) on phenetic and "cladistic" analyses. In the latter analysis, only a very small number of characters (17, and later 12) were used, some of which had states that did not show clear discontinuities and were therefore artificially coded – see Cauwet (1976, vol. 1, p. 582); and also Roux *et al.* (1978). Not surprisingly, Cauwet's classification of the genus was highly questionable, and also does not agree with the present phylogenetic analysis.

Two new subgenera: *Bupleurum* and *Penninervia*

There has been considerable discussion on the need of using many molecular markers in phylogenetic investigation, and many criticisms have been directed against the use of trees obtained from single genes as if they represented the true phylogeny of the taxa, i.e. to use a "gene tree" as if it was a "species tree" (Doyle, 1992). The molecular study of *Bupleurum* is at the very beginning, and with the exception of the work of Choi *et al.* (1996) on 4 species of *Bupleurum*, the present work is the first systematic investigation of molecular data within the genus – previous works included a few DNA sequences from *Bupleurum* (see section 3.7), but their aim was to investigate generic and suprageneric relationships in the *Umbelliferae* – see e.g. Downie *et al.* (1998), and Plunkett *et al.* (1996a,b & 1997), and Valiejo-Roman *et al.* (1998). There is no doubt that more evidence from other genes are needed before a good estimation of the phylogeny of the genus can be achieved. Nevertheless, as the degree of confidence of the two major clades obtained from analysis of ITS1 and ITS2 (fast evolving genes) and the 5.8S subunit (very low

rate of mutation) is very strong, I therefore propose the subdivision of *Bupleurum* into two new subgenera:

- Subgenus *Penninervia* – a small and basal group, which includes, as the name suggests, all the species with pinnate-reticulate veined leaves; the species are either shrubs or perennial herbs.

- Subgenus *Bupleurum* – a large group formed by most of the species of the genus (including the type species *B. rotundifolium* – see section 10.3), which includes shrubs, subshrubs, and perennial and annual herbs, that typically have ± parallel-veined leaves.

Subgenus *Penninervia* includes all *Bupleurum* species with pinnate-reticulate leaves, but also, *B. rigidum*, a species whose presence in this group came as a complete surprise, as it is morphologically quite distinct from the other members. Nevertheless, *B. rigidum* has itself a unique type of venation (veins ± parallel, but all very thick), that is not found in any other species in the genus. There is no risk that the sequence of *B. rigidum* was the result of contamination, as 4 different samples, collected by myself in the wild, were independently sequenced producing virtually identical ITS sequences (notice in Figs 9.5 & 9.6 that branch lengths for *B. rigidum* samples were zero; two of the sequences have one unidentified nucleotide). Sequence confirmation was also obtained for *B. fruticosum* and *B. gibraltarium* (two samples were sequenced for each).

However, there is a possible explanation of why *B. rigidum* has an ITS sequence that clearly belongs to this group, while morphologically resembling some members of the other subgenus. Such an explanation is that hybridization has occurred in the past between members of the two subgenera, but, due to *concerted evolution* (see section 9.1.1B - DNA sequencing), only one of the "parental" sequences was kept in the evolutionary lineage of *B. rigidum*. However, this is a quite complex explanation and, at the present, there is no evidence whatsoever to suggest that this may have happened. It seems simpler to accept that there is only apparent morphological similarity (convergence/ parallelism) between the flowering stems and inflorescence of *B. rigidum* and those of some other perennial herbs of subgenus *Bupleurum*, such as *B. acutifolium* or *B. oligactis*.

B. angulosum (endemic to the Pyrenees) and *B. stellatum* (endemic to the Alps and Corsica), are also part of subgenus *Penninervia*. These two species are perennial herbs and morphologically very close (fundamentally the only difference between them is the degree of fusion of the bracteoles – see notes under *B. angulosum* in chapter 10). These species form what has been designated as section *Reticulata* (because of the "reticulate" venation of the leaves), and, despite the low bootstrap (64 %) and jackknife (61 %) support for this clade (see Fig. 9.4), the group is very likely to be monophyletic; the morphological similarities are such that it seems improbable that they would have arisen independently.

I was unable to sequence *B. foliosum* (DNA extracts were very degraded and PCR amplification failed in all attempts – see section 9.3.1). Nevertheless, I am also including *B. foliosum* in subgenus *Penninervia* (see section 10.4), because it is morphologically very close to *B. gibraltarium*, and it is most likely that sequencing of ITS will confirm the presence of the species in this subgenus.

The clades within subgenus *Penninervia* have low support and further research is needed to clarify the relationships between these taxa. If the main subdivision of the subgenus *Penninervia* shown in the strict consensus tree is confirmed (Fig. 9.4; see also the tree topologies in Figs 9.5 & 9.6), a group defined by the presence of pinnate-reticulate leaves would be paraphyletic. The species with pinnate-reticulate leaves are *B. angulosum*, *B. foliosum* (not sequenced, and so not in the trees), *B. fruticosum*, *B. gibraltarium* and *B. stellatum*. If so, the subdivision of subgenus *Penninervia* indicated in section 10.4 would create a paraphyletic group, '*Penninervia*', and a monophyletic group, '*Crassinervia*' (including a single species, *B. rigidum*). The fact that a classification based on pinnate leaves may be artificial is not surprising, as this character (state) is probably plesiomorphic, and so we will be using a symplesiomorphy (shared ancestral character) to form groups instead of synapomorphies (shared derived characters).

The main clades within **subgenus *Bupleurum*** are unresolved (see Fig. 9.4, and compare Fig. 9.5 to 9.6). The first species to branch out, *B. baldense* (an annual herb), shows a considerably long branch (44 steps), which suggests that this clade may be an artifact of the sampling; notice that boostrap support for the dichotomy is

low (63 %), and that jackknife did not even show the clade (meaning that support was lower than 50 %). Also, there is no evidence (no 'peculiar' morphological character) suggesting that *B. baldense* is evolutionary isolated within the genus. This problem may be resolved by adding more taxa; there are a few E Mediterranean annual species that may be more closely related to *B. baldense*.

Nevertheless, subgenus *Bupleurum* shows three groups that have strong clade support, and are likely monophyletic groups (see Figs 9.5-9.7):

- '***Perfoliata***': This group corresponds to what have been so far considered section *Bupleurum* (see also chapter 2), and is characterised by the absence of bracts and the presence of perfoliate upper leaves. Phylogenetic analysis confirms that this is a natural group (boostrap support - 99 %; jackknife - 97 %), as has long been regarded by taxonomists. However, the tree topologies suggest that the rank of this group should be lower than section. It will be interesting to sequence the other 4 species with perfoliate leaves to verify if they also fall in this group (*B. croceum* Fenzl, *B. heldreichii* Boiss. & Balansa, *B. lophocarpum* Boiss. & Balansa, and *B. schistosum* Woronow – all endemic to Turkey; see Snogerup, 1972).

- '***Trachycarpa***': This group has been so far considered a subsection of section *Isophyllum*. It includes annual herbs, with linear to linear-lanceolate leaves, and papillose fruits. However, it is not clear what are the affinities of *Trachycarpa* as in the most parsimonious trees it either appears as a sister group of the 'NW African origin' group or of the group formed by *B. ranunculoides* and *B. longifolium*.

- '**NW African origin' group**: All the endemic species in NW Africa are included in this group; and also 2 Iberian species (*B. acutifolium* and *B. fruticescens* subsp. *fruticescens* and subsp. *spinosum*), a species endemic to the Balearic Islands (*B. barceloi*), and the endemic Macaronesian taxa (*B. salicifolium* and *B. canescens* var. *handiense*). The non NW African taxa are however morphologically close to some of the endemics in the area. *B. acutifolium* and *B. barceloi* are clearly allies of *B. oligactis*; while *B. fruticescens* subsp. *spinosum* also occurs in NW Africa. Furthermore, *B. salicifolium* is undoubtedly close to *B. canescens*. Another non NW African species that was not included in this work, but that is likely to belong to this group is *B. dianthifolium* Guss., a subshrub endemic to the island of Marettimo, near

Sicily, because it has morphological affinities to some of the taxa in the group, such as *B. acutifolium* and *B. barceloi*.

Another interesting fact in the 'NW African origin' group is that ITS presented a generally low nucleotide variation in the group (see Fig. 9.5 & 9.6 to see branch length), with some clearly distinct species having identical sequences (e.g. *B. benoistii* and *B. lateriflorum*). This may be an indication that the group has recently radiated. Morphological characters may have changed drastically (single mutations in 'morphological' genes may cause considerable changes in the phenotype), but there has been no time for mutations to accumulate in the genes, which change in a more gradual manner. In a recently evolved group, sequences will show very little variation, and are then poor for delimiting species (Bateman, 1999). However, *biased gene conversion* may also explain the maintenance of a particular sequence against new variants. If two different types of repetitive sequences are present in the genome (the 'original' and a new variant that have arisen by mutation), one of them will gradually substitute the other by *concerted evolution* (see section 9.1.1B). Theoretically, the chances are of 50 % for either of them to be conserved. But biased gene conversion towards one of the two different sequences have been detected in nuclear ribosomal DNA of some organisms, and in this case only one of them will be preserved independently of their initial dosage in the genome – see Hillis *et al.* (1991). So, if there is a 'tendency' to keep the original sequence against new variants, ITS will not accumulate changes between species. However, the latter explanation seems more unlikely, and the general pattern is that ITS does tend to diverge between different species (i.e. between taxa that no longer interbreed).

The group formed by *B. ranunculoides* and *B. longifolium* is also strongly supported in the analyses (bootstrap support 98 %; jackknife 96 % – see Fig. 9.4). There are undoubtedly morphological affinities between the taxa, but to confirm the monophyly of the group, I would like to see more taxa added to the analysis, especially other perennial herbs from Asia, such as *B. longicaule* Wall. ex DC., *B. candollei* Wall. ex DC., and taxa of the complex '*B. falcatum*' group.

Another interesting fact shown by ITS analysis is that annual habit seems to have evolved at least twice in the genus: **1)** In the clade including basically annuals

B. rotundifolium, B. gerardii, B. praealtum, B. odontites (the only perennial herbs are *B. falcatum* and *B. mundii*); *B. baldense* may belong to this clade. **2)** In the *Trachycarpa* group which includes only annuals.

Species delimitation in *Bupleurum*

The ITS analyses confirm that *B. fruticescens* and *B. spinosum* are a single species, as the sequences for the Iberian material were all identical. I had already reached this conclusion by the study of morphological variation in herbarium material, which was further supported by observations during field work in Spain (see *B. fruticescens* in chapter 10). However, the sequences obtained from the N African '*B. spinosum*' showed several differences in relation to those of the Iberian material of *B. fruticescens*. This is explained by the fact that Iberian and N African populations are no longer interbreeding, which is necessary to maintain the identity of ITS sequences within a species by the mechanisms of concerted evolution (see section 9.1.1B - DNA sequencing; and also Elder & Turner, 1995).

Note: The low bootstrap value (65 %) shown for the clade of the Spanish *B. fruticescens* and '*B. spinosum*' (see Fig. 9.4), which have identical sequences, reflects the fact that there is very little difference between the sequences of these taxa and those of *B. acutifolium* (Spain) and *B. barceloi*. In this situation, several of the bootstrap [pseudo] replicates will show identical sequences for the 4 taxa, because the random sampling of characters from the data matrix – see section 9.2.9 – will sometimes fail to 'pick up' the 2-3 characters (from a total of 615) that show nucleotide variation among these taxa. The same situation occurs in the clade formed by *B. salicifolium* Acc. Nos 29 & 294.

ITS sequences also confirmed my decision based on the study of herbarium material to regard *B. bourgaei* as a synonym of *B. ranunculoides* (see notes under *B. ranunculoides* in chapter 10). *Bupleurum bourgaei* has been so far considered an endemic of the mountains of SE Spain. The sample of *B. ranunculoides* Acc. No 297 (province of Jaén), correspond to material regarded as *B. bourgaei*, but the sequence of this sample only differs in one equivocal nucleotide (see Fig. 9.3) to that of *B. ranunculoides* Acc. No 296, from the province of Burgos in NE Spain.

There are minor morphological differences between the material of the two populations of *B. acutifolium* (Portuguese and Spanish); but neither of these are reliable as they show considerable plasticity. However, the ITS sequences obtained for the two populations differ considerably (the sequences for each population were confirmed – see section 9.3.1). The sequence of the Portuguese material shows a fairly large deletion in ITS1 (35 bp), which is likely the result of a single mutation, but there are 14 other single nucleotide differences (see Fig. 9.3); far too many for samples of a single species (normally only 0-4 differences are found within a species). The geographic distance between the two populations is quite large (see *B. acutifolium* in chapter 10), and inbreeding has probably not occurred for a considerable time. But still there are too many differences in the sequences, and the sequence of the Spanish population is remarkably close to that of *B. fruticescens* (a Spanish 'neighbour', which is clearly a distinct species). Therefore, further research is needed to ascertain if the two populations of *B. acutifolium* correspond to two different species.

Another taxon whose samples showed considerable differences was *B. balansae*. But in this case, I may be using inadequate morphological characters to recognize the taxon. The main character that has been used for identification is that flowers and fruits are sessile or subsessile. However, there is too much variation in shape and length of leaves and even in prominence of veins, and is possible that I am mixing under the same name two different species – see also the comments under *B. balansae* in chapter 10. Further research is needed on the different populations of these 'shrubs with sessile flowers' for a better morphological delimitation.

One of the sequences obtained for *B. gerardii* (Acc. No 307) does not form a group with the others. So there seems to be a problem of species delimitation, which is not really a surprise, considering the difficulties I experienced distinguishing *B. gerardii* from *B. praealtum* (see notes under these species in chapter 10). Notice that in the tree of Fig. 9.6 (one of the maximally parsimonous tree, i.e. one possibility of how evolution may have happened), *B. gerardii* 307 appears as a sister taxon of the clade including *B. gerardii* and *B. praealtum*. One of the possibilities is that hybridization has occurred between *B. gerardii* and *B. praealtum*, which would explain the problems of morphological characterization of the taxa. A second

possibility is that I am mixing under *B. gerardii* two different species. A more detailed study of *B. gerardii* is necessary, including material from the rest of the area of distribution (Europe and W Asia), and also other allied species, such as *B. commutatum* Boiss. & Balansa, *B. praealtum* and *B. trichopodum* Boiss. & Spruner.

Relationships of *Bupleurum* with other genera

ITS sequences have been obtained for *Bupleurum* by earlier workers (Choi *et al.*, 1996; Lee & Rasmussen, 1998; and Valiejo-Roman *et al.*, 1998), and some difficulties were experienced obtaining unambiguous alignment with sequences of other *Apioideae* taxa, even with other basal genera in the subfamily (see Valiejo-Roman *et al.*, 1998). However, the main reason for the problematic alignment appears to be that none of these studies included sequences from the basal group (*Penninervia*) in *Bupleurum*. Sequences of these taxa are not so disparate from those of other basal *Apioideae* genera, such as *Heteromorpha* (see sequence alignment in Fig. 9.3 and compare, for example, the sequences of *Heteromorpha arborescens* and *Bupleurum fruticosum*). ITS sequences of *Anginon* (obtained for the first time in the present work) are however very distinct from *Bupleurum*, but also, surprisingly, from *Heteromorpha*. Previous molecular work have shown *Anginon* and *Heteromorpha* as closely related genera – see the results of the analysis of the chloroplast genes, *rbc*L and *mat*K, in Plunkett *et al.* (1996a,b & 1997), and of the *rpo*C1 intron, in Downie *et al.* (1998).

The various studies carried out have confirmed the genus *Bupleurum* as a basal ('primitive') genus in subfamily *Apioideae*, but considerable research is still needed concerning the relationships of *Bupleurum* and other basal genera in the subfamily.

Geographical distribution

An interesting fact of the ITS analysis in *Bupleurum* is that several clades are remarkably congruent with the geographical distribution of the taxa. The most clear case is the large group I have designated of 'NW African origin', a clade which includes all the endemics to NW Africa. Some of these endemics are

morphologically quite distinct, and they have not been so far classified as a single group. As said before nucleotide variation is generally small within the 'NW African origin' group and so only a few changes support the branches, but two of its groups merit some attention:

1) The Macaronesian endemics, *B. salicifolium* (Madeira and Canary Islands) and '*B. handiense*' (= *B. canescens* var. *handiense* – E Canary Islands) appear together, and also with *B. canescens* s. str. (endemic to S Morocco) – these taxa are also morphologically close (see chapter 10). The latter taxon occurs in an area of Morocco that is recognised by some authors as 'the Macaronesian enclave' in NW Africa (Sunding, 1979, p. 14-15). So, ITS variation shows a '*Macaronesian clade*' with moderate branch support [bootstrap support is good = 89 %, but the branch is only 2 steps long]. The ancestor of this clade appears to have originated in NW Africa, with his descendants having later colonised the islands. *B. salicifolium* may have evolved on the islands as is only present there; but the origin of *B. canescens* is more doubtful as it could either be the Canary Islands or Morocco. An interesting fact is that these taxa have managed to disperse over the ocean, despite that their fruits lack special adaptations to dispersion (most *Bupleurum* fruits do not have them). Nevertheless, seeds of this type can be dispersed accidentally by birds over long distances or 'oceanic barriers' (Bramwell, 1985).

2) The clade including *B. acutifolium* (Spanish population), *B. fruticescens* and '*B. spinosum*' (Spanish populations), and *B. barceloi* (endemic to the Balearic Islands), is strongly supported (bootstrap = 98%; jackknife = 96 %), and has a fairly reliable branch length (6 steps). This suggests that the most direct ancestor of *B. barceloi* may have originated from the Iberian peninsula, probably from the Spanish mainland.

Two clades of subgenus *Bupleurum* include species that have essentially an Eurasian distribution: **a)** the clade including '*Perfoliata*' and the groups of *B. falcatum* and *B. gerardii*; and, **b)** the clade of *B. ranunculoides/ B. longifolium*. *Bupleurum lancifolium* and *B. odontites* (included in clade 'a') occur in N Africa (see maps in chapter 10), but their original (native) area of distribution is not properly known as both species have been introduced as ruderals or weeds in cultivated land.

The only exception in this Eurasian distribution is *B. mundii*, a species endemic to S Africa, which closest relative in this analysis is *B. falcatum* (there are also morphological affinities between these two species). In section 10.4 (synopsis of the classification), this species group (including also *B. baldense*) is designated as 'Eurasian heterogeneous macrogroup'; the term 'heterogeneous' is used to remark that this is probably an artificial group, as clades were not clearly resolved by ITS analysis.

Centre of origin of the genus *Bupleurum*

ITS analysis suggests that the genus *Bupleurum* may have originated somewhere in the W Mediterranean.

First, the basal (more 'primitive') clade in *Bupleurum* is formed by species that basically only occur in the W Mediterranean. *Bupleurum fruticosum* is the only species with a distribution that extends across all the Mediterranean, but this can easily be explained as the result of subsequent dispersion. Also, the shrubby plants of the genus are restricted to the W Mediterranean (the only exception, again, is *B. fruticosum*). This is particularly relevant if woodiness is regarded as an ancestral (plesiomorphic) state, which appears to be supported by wood anatomy in *Bupleurum* (Alexei Oskolski, Botanical Museum of St. Petersburg – personal communication). Also, the W Mediterranean is undoubtedly more rich in the 'basic' morphological patterns found in *Bupleurum*. The E Mediterranean has high diversity in annual species (see e.g. *Flora of Turkey* – Snogerup, 1972), but it is likely that they have evolved more recently. ITS analysis indicates that annual habit is a derived state (possibly having evolved more than once in the genus). Shorter life cycle increases the chances of evolutionary change, so a shorter period of (geological) time is required to explain diversity in annual plants. The morphological variation of *Bupleurum* in the rest of Asia and N America seems to revolve around a more limited set of patterns of mostly perennial herbs (*B. falcatum*, *B. ranunculoides* and *B. longifolium* 'morphotypes'). For example, the only species naturally occurrying in N America, *B. triradiatum* Adams (syn. *B. americanum* Coult. & Rose) is remarkably similar to *B. ranunculoides* (see e.g. Húlten, 1968, p. 698).

Geological time and *Bupleurum*

Unfortunately, *Bupleurum* fossil record is virtually non existent. Pollen fossil of what appears to be *Bupleurum* have been recorded for geological strata of the Parisian Basin of the early Eocene (c. 50 million years ago) – Gruas-Cavagnetto & Cerceau-Larrival (1978). However, even if we are to believe that the pollen is indeed of *Bupleurum*, very little can be said other than maybe *Bupleurum* had already evolved at the time. With no evidence whatsover, Cauwet (1976, vol. 1, p. 267, 297), argued that as *Bupleurum* is very "ancient", it was "reasonable" to suggest the "appearance" of the genus in the early Albian during the Cretaceous (c. 100 million years ago).

If *B. salicifolium* has been found to be a 'primitive' taxon with no close continental relatives (i.e. a *palaeoendemic*), we could have thought that its arrival (or that of its ancestor) to Madeira would have happened at a time when this island was far closer to the African continent (at present Madeira is 630 km W of the cost of Morocco). But, *B. salicifolium* is clearly a *neoendemic* species, and, the ITS sequences of the samples from Madeira and Canary Islands were found to differ only in one nucleotide, which indicates an even more recent arrival (as mentioned above, birds may transport seeds accidentally between different islands and over long distances).

Another neoendemic is the disjunct species *B. mundii* (endemic to S Africa), which is therefore not a remnant species from a past larger distribution of *Bupleurum* in Africa. This has been questioned before, in particular considering the relationships of woody (basal) *Umbelliferae* genera, most of which only occur in C & S Africa (Burtt, 1991; Downie & Katz-Downie, 2000, *in press*; and Cerceau-Larrival, 1971).

At the moment, the only possibility left to estimate a time of evolution of *Bupleurum* appears to be the use of the **'molecular clock'**. For detailed discussion on 'molecular clocks', its controversies and limitations, see Li (1997, p. 1-6, 215-235) and Hillis *et al.* (1996b, p. 531-540).

The *molecular clock hypothesis* assumes that for a particular macromolecule (nucleic acid or protein) the rate of evolution (mutation) is approximately constant in different lineages of organisms. So, after measuring molecular divergence between

different taxa and assuming a certain rate of mutation, one can estimate a time of evolution for a particular taxon or group.

However, much controversy has been generate around this subject, especially because there seems to be far too much variation in rates of mutation of single genes among different organisms. This variation appears to be due to differences in metabolic rates, the efficiency of DNA repair, and generation time of organisms (Li, 1997, p. 228-231) – [*generation time* is, in this case, the period of time that goes from the production of seed, its germination, the full development of a plant, to the eventual production of new seeds].

There are also considerable problems on calibration of the 'molecular clock' (i.e. the determination of the mutation rate), even when dated fossil is available. Also, the time intervals of confidence can be so broad that they may not provide any meaningful information (Moritz & Hillis, 1996, p. 10-11; Hillis *et al.*, 1996b, p. 534-539).

It has been argued that as closely related taxa have similar metabolism, the rates of mutation of their genes should also be similar. So, if the molecular clock is calibrated for each gene in each taxonomic group, we can more reliably predict time of divergence. However, it is not unusual that close taxa present different generation times; for example, many plant genera include both annuals and perennials – the case of *Bupleurum*.

If we look again to the trees obtained from ITS analysis in *Bupleurum*, especially to those where branch length (number of nucleotide changes) is shown (Figs 9.5-9.6), we may notice that the herbaceous taxa, particularly annuals, show in several cases longer branches than their perennial relatives [compare for example the taxa in the clade of herbaceous species including *B. gerardii* and *B. rotundifolium* (which are annuals), to those in the 'NW African origin' group (all perennial – many shrubs and subshrubs)]. Not taking in consideration generation time can lead to the conclusion that a clade of annual or herbaceous species would have evolved much earlier in time (as it showed more mutations) than a clade that contained essentially perennials or shrubs. But the case may be exactly the opposite, and obviously any geological dating would be incorrect. A major problem is that we will never know the generation times of the different ancestors in a particular lineage, and how the

rates of mutation may have been modified by other factors, such as drastic environmental changes.

There is no doubt that confidence on the use of molecular data to time evolutionary divergence is low. But the use of the 'molecular clock' from several genes, providing independent estimates of time of evolution, may still bring some relevant information, in particular for taxa where data fossil is scarce or non existent.

9.5 Further work

It will be important to enlarge the number of samples and taxa sequenced for ITS in *Bupleurum*, in particular to include E Mediterranean and Asian taxa. For example, it would be interesting to verify if *B. foliosum* is placed in subgenus *Penninervia*, or *B. dianthifolium* in the 'NW African origin' group.

It is essential to sequence other genes to confirm and to improve the resolution of the present phylogenetic analyses. Sequences of several other genes, with various rates of mutations, have been used in molecular systematics – for a review, see Soltis & Soltis (1998). Sequencing a gene with slower rate of mutation than ITS may help to resolve some of the clades in *Bupleurum*; e.g. the chloroplast genes *tnr*L and *mat*K have also been useful at generic and infrageneric levels.

There are other methods of molecular analysis that can provide valuable data for systematic research – see Hillis *et al.* (1996b, p. 516-521); see also section 9.1.1. For example, restriction site analysis of chloroplast DNA (see RFLPs) has been a good source of data for phylogenetic analysis, in particular at interspecific level and below (see Jansen *et al.*, 1998).

The 'NW African origin' group showed very low nucleotide variation in the ITS region. At present, the ITS spacers (ITS1 and ITS2) are the fastest evolving genes sequenced for phylogenetic analysis [other fast genes are known, but sequence homology or alignment have been problematic]. Therefore, to resolve the relationships of the taxa in this group, we will need methods of analysis that can detect molecular variation at lower levels. Isozymes, AFLPs and microsatellites (see section 9.1.1) are techniques that have been particularly useful to detect molecular polymorphism at population level and below.

10. Formal Taxonomy

10.1 Introduction

This taxonomic treatment refers to species of *Bupleurum* L. occurring in the W Mediterranean and Macaronesia regions as delimited in chapter 4. The ranges of several of these species extend beyond the defined geographical limits, and present taxonomic problems well beyond the scope of this study, and, are therefore, not discussed here in detail (e.g. the polymorphism of *B. falcatum* L.). It needs to be emphasised that the variation that happens outwith the studied area, in any particular taxon, has not been ignored. My excursions into this 'foreign' material have made me look critically at my judgements on the taxa and helped to make them more objective in a broader generic context. There is no doubt that much further research is needed for several of the taxa.

For many of the well-known Iberian/European taxa, my work has often confirmed the decisions of previous authors. But even in these cases, there was still matters to debate at the specific level (and still there is!). I hope that the extra information I have gathered for these apparently 'easy' species will be of use to other botanists not so familiar with the group. My account of the genus has been already submitted for *Flora iberica* vol. 10.

In contrast, the study of the NW African taxa should be regarded as the first attempt of a complete study of *Bupleurum* in the area. The taxonomic review of Cauwet (1976) included all NW African taxa, but did little to improve the situation. Cauwet basically accepted most of the previous published names with little questioning. Her groups were 'pre-defined' without clear reasons (no comparable or clearly distinct characters where used). A key to identification of the species was presented only for Cauwet's subgenus *Tenoria* (even then 2 of the species were left out of the key: *B. antonii* Maire and *B. montanum* Coss.). Furthermore, the morphological descriptions provided did not distinguish most of the species. Her subsequent studies concentrated exclusively on particular species, e.g. *Bupleurum atlanticum* Murb. (Cauwet & Carbonnier, 1975, 1976, 1977) and *B. salicifolium* R.Br. ex Buch from Macaronesia (Cauwet & Sunding, 1981).

My own account is aimed at providing a much better view of *Bupleurum* in NW Africa, explaining more clearly how to identify the 'easy/good' species, and leaving the appropriate question marks on those that need further research.

The taxonomy presented here is based primarily on morphological characters. It could almost have been 'exclusively on morphology', but the only infrageneric groups delimited, two new subgenera (*Bupleurum* & *Penninervia*), are based essentially on molecular characters (ITS gene sequencing – see chapter 9). This main division shown by molecular data is almost completely supported by morphological characters, as only *Bupleurum rigidum* L. disturbs this view. The proposed new subgenus *Penninervia*, as the name suggests, contains all pinnately veined species in the genus (but also *B. rigidum*, that has itself a most unique type of venation). The type subgenus *Bupleurum*, as defined here, includes the rest of the genus and all the typically parallel-veined species, i.e. the vast majority of the species known.

Some informal groups are also presented (see section 10.4). They are supported by both morphological and molecular data (except the 'heterogeneous' subgroup 2). However, the data obtained are not conclusive and therefore no formal taxonomic rank has been attributed. For more detailed discussion on the subgeneric and informal divisions see chapter 11 & also chapter 9 for details of molecular data.

10.2 Material & methods

The information presented here for all the species largely follows that required for the *Flora iberica* project, plus additional information not published in the *Flora*, e.g. types. However, after several modifications in style, and addition of extra details (e.g. maps) to this account, the original format for *Flora iberica* is hardly recognisable.

The following is the list of information given for each taxon, arranged as it appears in the text: **a)** *Type*(s) and *type locality* (for many species this represents several localities cited in the protologue, as a holotype was in general not designated); **b)** *synonyms* (in chronological order); **c)** *name origin* (derivation of the specific name); **d)** *illustrations* (when available, in chronological order); **e)** *morphological description of the species*; **f)** *chromosome numbers* (from the literature); **g)** *ecology* and *altitudinal range* (information obtained from herbarium

specimens and/or the literature); **h)** *flowering time* (includes also fruiting time); **i)** *World distribution* and *distribution in the W Mediterranean and Macaronesia* (plus map of distribution and notes); **j)** *vernacular names* (from the literature); **k)** *representative specimens* (selection of herbarium material studied), and **l)** supplementary notes: *conservation status* (if the taxon is endangered, in decline or very rare), *critical taxonomic notes* (e.g. pointing out characters that facilitate identification), *typification notes*, and *medicinal* and *other uses* (when known).

Detailed information on plant material studied is given in chapter 6 (section 6.1.1). Also, in order to study type material of the species of Linnaeus, all *Bupleurum* specimens in LINN and in the herbarium of Clifford (BM) were examined (1998). Also, IDC microfiches of the herbaria of Burser (UPS) and Linnaeaus (S) were consulted.

Unless otherwise stated, all herbarium material cited in the text has been revised by myself. Considering the total amount of herbarium material revised, only a small selection of the specimens studied is cited in the text (I have a record of a much larger number of specimens). Only in one case was all the material studied cited: *B. subspinosum* Maire & Weiller – I only found 8 specimens, 5 of which are syntypes!

10.2.1 Basic taxonomic references

The 'basic' references for *Flora iberica*, relevant to *Bupleurum*, are: Bolòs & Vigo (1990), Cadevall (1919), Coutinho (1939), Franco (1971), Knoche (1922), Merino (1905), Tutin (1968), Willkomm (1893), and Willkomm & Lange (1874). In relation to Iberian and Balearic taxa, other publications were also consulted: Barceló (1979), Beckett (1988), Bolòs *et al.* (1990), García Martín (1987), Gómez *et al.* (1996), Rollán (1985), Romo (1994) & Sagredo (1987).

Concerning NW African taxa, the following are the most relevant works: Battandier (1910), Battandier & Trabut (1889), Emberger & Maire (1941), Jahandiez & Maire (1932, 1934), Négre (1962), Ozenda (1958), Panelatti (1959), Pottier-Alapetite (1979), Quézel & Santa (1963), and Sauvage (1961). The most relevant

references for Macaronesian taxa are: Bramwell & Bramwell (1974, 1990), Hansen & Sunding (1993), Heywood (1973), Kunkel (1975), and Press & Short (1994).

The *Index Kewensis* (1893-cont.) and the *Kew Record* (1974-cont.), essential sources of taxonomic references, were also consulted. As general references on description of species, ecology, distribution and other information, the following Floras were also consulted: Jafri (1985), Rechinger & Snogerup (1987), Snogerup (1972) and Stace (1997). Chromosome numbers were obtained from Cauwet (1979a), Goldblatt (1981-1988), and Goldblatt & Johnson (1990-1998). The names of the phytosociological associations (indicated under ecology), were obtained mainly from Bolòs & Vigo (1990) and Bolòs *et al.* (1990). Vernacular names were obtained from Ceballos (1986), Morales (1992), Morales *et al.* (1996), and Quézel & Santa (1963).

Abbreviations and publication dates of books follow, when possible, *Taxonomic Literature* (Stafleu, 1967; Stafleu & Cowan, 1976-1988; & Stafleu & Mennega, 1992-1998), and also Heller (1958), the latter being very useful for pre-Linnaean books. When no standard abbreviation is available (e.g. recent publications), a similar style of abbreviation to that of *Taxonomic Literature* is used. Abbreviations of journals and other periodicals follow the *Botanico-Periodicum-Huntianum* of Lawrence *et al.* (1968), and its *Supplementum* of Bridson & Smith (1991). Herbarium abbreviations follow Holmgren *et al.* (1990). Authors names are according to Brummit & Powell (1992). For correct use of botanical terms and understanding of descriptions in Latin, Stearn (1983) was of invaluable help.

10.2.2 Typification: general notes

Concerning Linnaean species, the information on what is original material and which species had already been typified was kindly provided by the Linnaean Plant Name Typification Project (Natural History Museum, London).

Most of the NW African endemic taxa still required typification as holotype was generally not indicated in the protologue. Panelatti (1959) used the expression "spécimen type" when citing the herbarium material she used in her anatomical studies. However, it appears that her intention was not to select the type, but only to remark that she had used for some species (what she considered to be) type material.

For example, in some cases the specimen cited was indeed a syntype (e.g. for *B. album* Maire), but others were not type material (e.g. for *B. faurelii* Maire and *B. mesatlanticum* Litard. & Maire). Another example: Panelatti indicated as the "spécimen type" of *B. montanum* Coss. a specimen in Alger, when the type should be selected from Cosson own herbarium in Paris (i.e. from the material the author would have used to describe the species). Furthermore, in the case of *B. oligactis* Boiss. she explicitly said that she did not see the specimen, and that a sample was sent to her from Geneva. Therefore, I do not regard any of these citations as typifications.

10.2.3 The maps of distribution

All maps presented in this section were graphically manipulated using the program Adobe Photoshop 4.0 (Adobe Systems, Incorporated – version for Microsoft Windows 95), after drawings or photocopied images were scanned (HP Deskscan II, version 2.7 – Hewlett Packard Co.). The map of the Iberian peninsula (Fig. 10.1) was extracted from *Flora iberica* (Castroviejo *et al.*, 1986-continuing), but it has been graphically modified. The map of NW Africa (Fig. 10.2) is the result of the 'fusion' of 4 maps of the following floristic studies: **a)** Morocco: *Catalogue des Plants du Maroc* (Jahandiez & Maire, 1931), and *Floristic Biodiversity of Northern Morocco* (see Valdés, 1994). **b)** Algeria: *Nouvelle Flore de l'Algérie* (Quézel & Santa, 1962) – areas in the map were simplified. **c)** Tunisia: *Flore de la Tunisie* (Pottier-Alapetite, 1979). The map of Macaronesia was based on maps easily available: e.g. in *The Times Atlas of the World* (several editions). For explanation of abbreviations used in the maps or in the main text, see Figs 10.1-10.3.

For the Iberian Peninsula, the Balearic Islands and Macaronesia, a very large and representative amount of herbarium material was revised. Therefore, the distribution indicated in the maps is based on specimens seen by myself. Only in a few cases I did not see material for a province or island cited in the literature. In this case, the abbreviation of the province/island is placed in brackets, and a lighter shading is used for the area in the map. The references for these citations are given in notes, most of which were obtained from *Archivos de Flora iberica* (see Velayos *et al.*, 1991-1993).

However, NW African collections are much smaller, and the material I had available for study was not representative of the real distribution of the taxa, especially for Algeria and Tunisia – the collections for Morocco have been recently enlarged with expeditions organized by Reading and Sevilla Universities, in connection with the project of the *Floristic Biodiversity of Northern Morocco* (see Valdés, 1994). Therefore, the distribution given for NW African taxa is only partially supported by revised herbarium material, and is supplemented by references in the following works: Emberger & Maire (1941), and Jahandiez & Maire (1932, 1934) for Morocco; Quézel & Santa (1963) for Algeria; and Pottier-Alapetite (1979) for Tunisia. In the NW African map, a lighter shading is used only if the taxa is believed to be very rare in the area, or if the occurrence is doubtful.

For all the area in study, the Gazetteers (Official Standard Names, US. Board on Geographic Names, Washington, DC.) of Algeria, Morocco, Portugal, Spain and Tunisia were extremely helpful to find and position in the maps the names of localities cited in herbarium specimens.

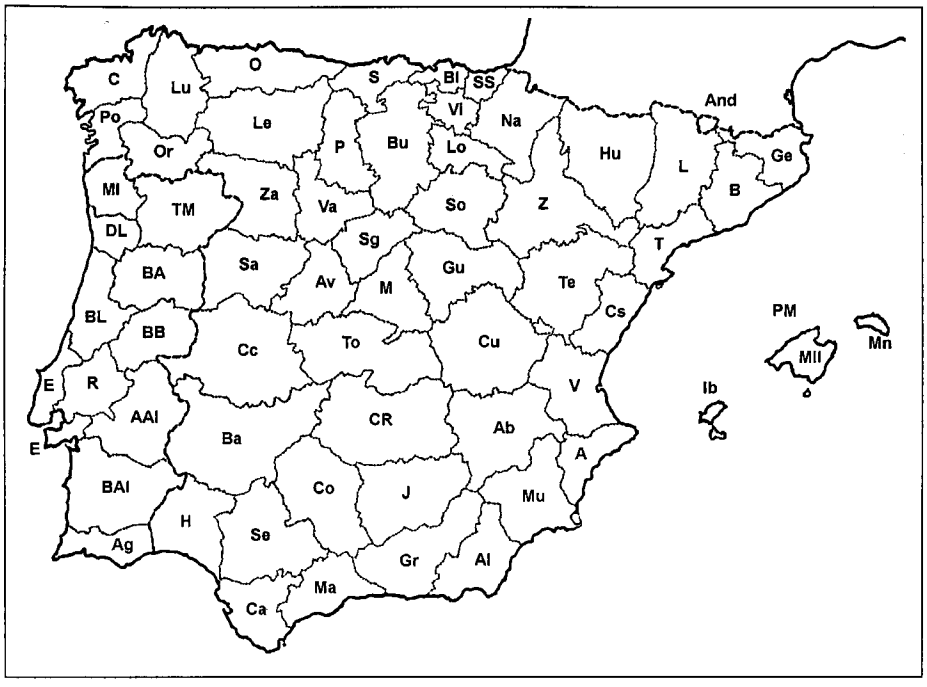


Fig. 10.1 – The Iberian Peninsula provinces & the Balearic Islands.

***Andorra*: And.**

***Spain*: A-** Alicante; **Ab-** Albacete; **Al-** Almería; **Av-** Ávila; **B-** Barcelona; **Ba-** Badajoz; **Bi-** Vizcaya (Bilbao); **Bu-** Burgos; **C-** La Coruña; **Ca-** Cádiz; **Cc-** Cáceres; **Co-** Córdoba; **CR-** Ciudad Real; **Cs-** Castellón; **Cu-** Cuenca; **Ge-** Gerona (Girona); **Gr-** Granada; **Gu-** Guadalajara; **H-** Huelva; **Hu-** Huesca; **J-** Jaén; **L-** Lérida (Lleida); **Le-** León; **Lo-** La Rioja (Logroño); **Lu-** Lugo; **M-** Madrid; **Ma-** Málaga; **Mu-** Murcia; **Na-** Navarra; **O-** Asturias (Oviedo); **Or-** Orense; **P-** Palencia; **PM-** Balearic Islands **[MII-** Mallorca; **Mn-** Menorca; **Ib-** Ibiza **]**; **Po-** Pontevedra; **S-** Cantabria (Santander); **Sa-** Salamanca; **Se-** Sevilla; **Sg-** Segovia; **So-** Soria; **SS-** Guipúzcoa (San Sebastián); **T-** Tarragona; **Te-** Teruel; **To-** Toledo; **V-** Valencia; **Va-** Valladolid; **Vi-** Álava (Vitoria); **Z-** Zaragoza; **Za-** Zamora.

***Portugal*: AAl-** Alto Alentejo; **Ag-** Algarve; **BA-** Beira Alta; **BAl-** Baixo Alentejo; **BB-** Beira Baixa; **BL-** Beira Litoral; **DL-** Douro Litoral; **E-** Estremadura; **Mi-** Minho; **R-** Ribatejo; **TM-** Trás-os Montes e Alto Douro.

Note: In the text, abbreviation in brackets, e.g. (Ca), means that I have not seen material for the province, but that there is a citation in the literature for the area.

[From ***Flora iberica***, Castroviejo *et al.* eds (1986-cont.) – graphically modified].

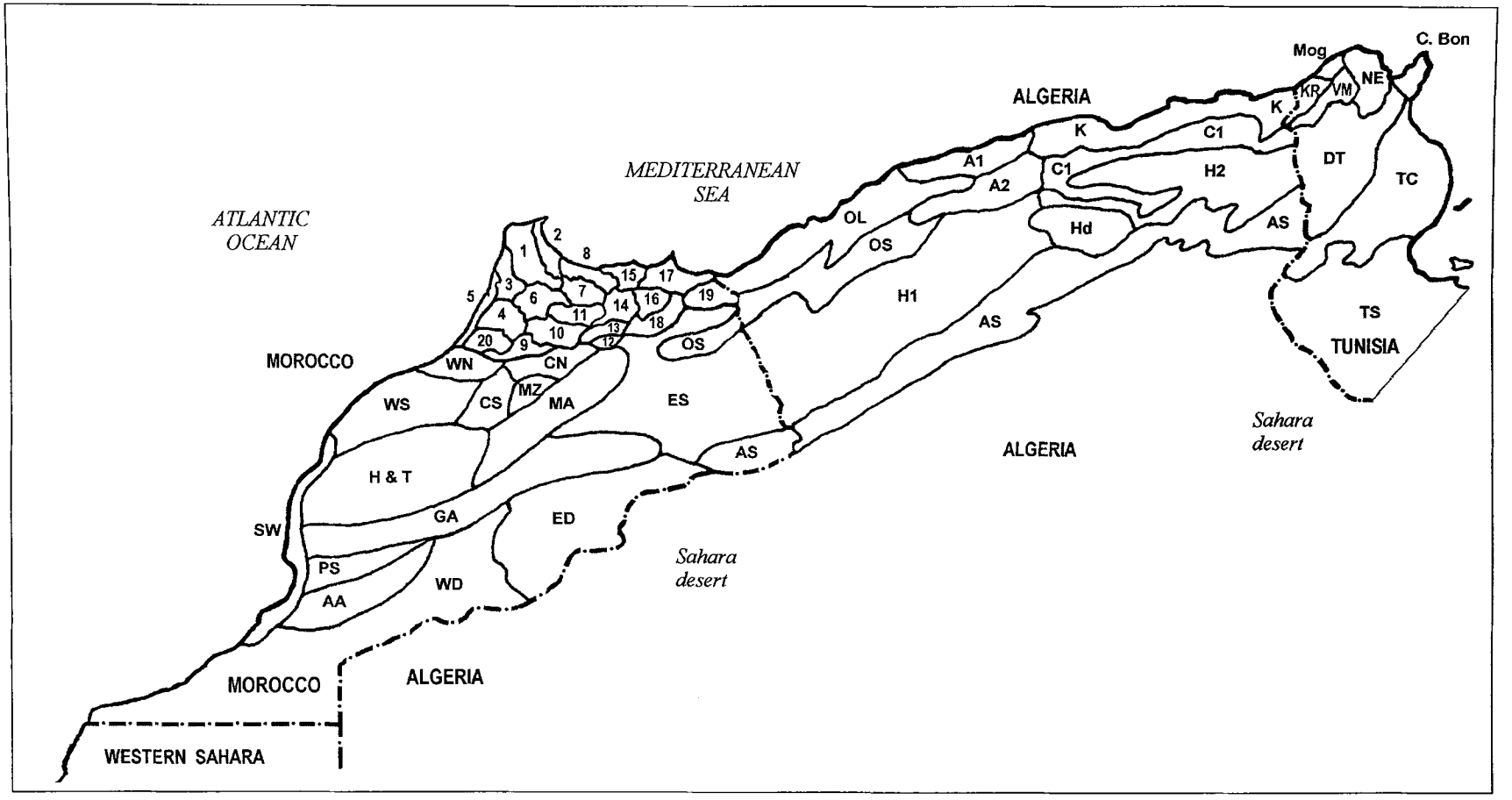


Fig. 10.2 – NW African geographical divisions: **1-** Tanger area; **2-** W Rif; **3-** Loukkos; **4-** Gharb; **5-** Atlantic Coast; **6-** Ouezanne; **7-** C Rif; **8-** Targuist; **9-** Zerhoun; **10-** Pre-Rif; **11-** High Ouerrha; **12-** Tazzeka; **13-** Tsoul; **14-** Aknoul; **15-** Imzovène; **16-** Kert Ganc; **17-** Gareb; **18-** Guercif; **19-** Beni-Snassen; **20-** Mamora Forest. **A1 & A2 -** Alger area (**A1**- Littoral; **A2**- Tell Atlas); **AA-** Anti-Atlas; **AS-** Saharan Atlas; **C1-** Constantine Tell; **C. Bon-** Cape Bon; **CN-** NC Morocco; **CS-** SC Morocco; **DT-** Dorsal Tunisia; **ED-** E Moroccan desert; **ES-** E Moroccan steppe; **GA-** Great (High) Atlas; **H1-** Alger-Oran High Plateau; **H2-** Constantine High Plateau; **Hd-** Hodna Plateau; **H & T-** S Moroccan steppe (Haouz & Tadla); **K-** Kabylie; **KR-** Kroumirie; **MA-** Moyen (Middle) Atlas; **Mog-** Mogod; **MZ-** Zaïan mountains; **NE-** NE Tunisia; **OL-** Oran Littoral (Beni-Snassen); **OS-** Oran mountains; **PS-** Sous Plain; **SW-** "Macaronesian Moroccan" sector (Cape Cantin to Ifni); **TC-** C Tunisia; **TS-** S Tunisia; **VM-** Mejerdah Valley; **WD-** W Moroccan desert; **WN-** Rabat to Casablanca; **WS-** Casablanca to Cape Cantin.

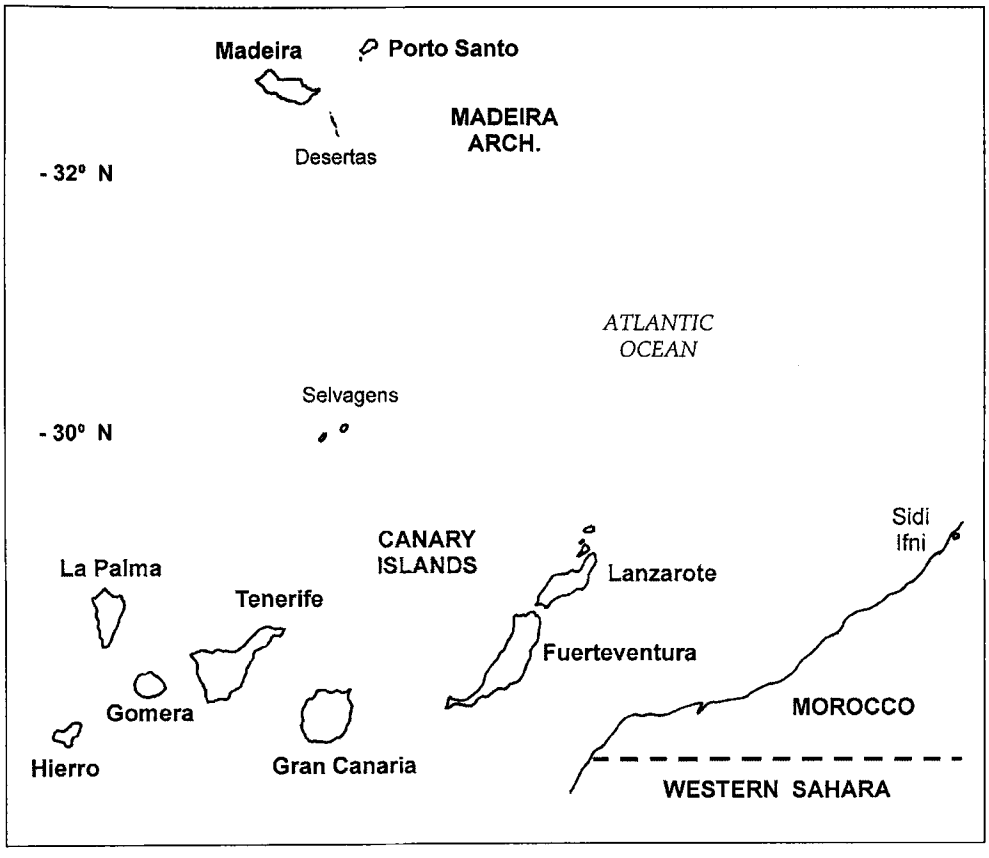


Fig. 10.3 – The Macaronesian Islands.

Canary Islands (*Spain*): Fuerteventura (**Fuertev.**); Gran Canaria (**G. Can.**); Gomera (**Gom.**); Hierro (**Hier.**); Lanzarote (**Lanz.**); La Palma (**Palm.**); Tenerife (**Tener.**).

Madeira Archipelago (*Portugal*): Madeira (**Mad.**); Porto Santo (**P. Sto**).

Note: Macaronesian Archipelagos where *Bupleurum* does not occur are not included. Abbreviations are used in the main text.

10.3 Generic description

Bupleurum L., *Sp. pl.* 236 (1753); *Gen. pl.*, ed. 5: 110 (1754).

Shrubs, subshrubs, or perennial and annual herbs, glabrous. *Cotyledons* linear, herbaceous, 1-veined, glabrous. *Leaves* simple, entire, with a very narrow marginal band (smooth or minutely serrulate), subsessile or ± amplexicaul (basal leaves sometimes attenuate into petiole of variable length, upper leaves very rarely perfoliate); pinnate-reticulate or more often parallel-veined, with or without secondary veins; glaucous, green or rarely with purplish colouration. *Inflorescence* a compound umbel, rarely compacted or reduced to apparently simple umbels; *bracts* present, very rarely absent or deciduous; *bracteoles* present, sometimes conspicuous. *Flowers* hermaphrodite, actinomorphic; *calyx teeth* absent (obsolete); *petals* 5, all similar, yellow or greenish-yellow, more rarely with purplish or pinkish tones, or extremely rarely whitish, with or without a darker mid-vein, apex inflexed (± adnate to the back of the petal), entire, bilobed or very rarely fimbriate, smooth at the top, sometimes slightly tuberculate; *stylopodium* ± flat. *Fruit* 2 mericarps, splitting at maturity, transversally isodiametric or slightly laterally or dorsally flattened, smooth, or more rarely tuberculate or papillose, only with primary ridges (5); ridges filiform, prominent or narrowly winged, very rarely inconspicuous, smooth or very rarely crenulate; vittae (oil ducts) present (1-4 per vallecula, 2-6 in commissure), rarely absent, sometimes with secondary oil ducts, 1 in each of the ridges, located externally to the vascular bundles.

Type species: *Bupleurum rotundifolium* L. – Herb. Linn. 335.1 (LINN!) - recommended as conserved type [*Taxon* 44(4): 612 (1995)].

The selection of the above type species was made by Hitchcock (1929); but it should be noted that the earliest designated type species is *Bupleurum rigidum* L. (Britton & Brown, 1913) – see *Taxon* 41(3): 572 (1992); & 36(2): 350-352, 363 (1987). However, *B. rotundifolium* has been adopted as the type in most recent treatments, and acceptance of the earlier type would cause major changes in infrageneric nomenclature. The proposal for conservation of *B. rotundifolium* (Report of Subcommittee 3C on Lectotypification of Linnaean generic names)

gained a small majority, with less than two thirds in favour [*Taxon* 41(3): 572 (1992)]. In 1995, the Committee for Spermatophyta: 43 voted to accept as conserved types all names listed in the report of the Subcommittee 3C, including *Bupleurum*.

10.4 Synopsis of the classification

For discussion of the classification see sections 9.4 and 10.1, and chapter 11.

I) Subgenus *Bupleurum*

Shrubs, subshrubs or perennial and annual herbs. *Leaves* ± parallel-veined, sometimes with thick midrib, but thick intramarginal vein absent.

Type of subgenus: *B. rotundifolium* L.

Group A - 'Eurasian heterogeneous macrogroup'

Annual or perennial herbs. Leaves herbaceous. Bracts present or absent. Fruits smooth or tuberculate.

Subgroup 1 - '*Perfoliata*'

Annual herbs. Leaves herbaceous, upper leaves perfoliate. Bracts absent. Fruits smooth or tuberculate.

1 - ***B. lancifolium*** Hornem.

2 - ***B. rotundifolium*** L.

Subgroup 2 - 'Eurasian heterogeneous group'

Annual or perennial herbs. Leaves herbaceous, subamplexicaul, sometimes the basal petiolate. Bracts present. Fruits smooth.

3 - ***B. baldense*** Turra

4 - ***B. falcatum*** L.

5 - ***B. gerardii*** All.

6 - ***B. odontites*** L.

7 - ***B. praealtum*** L.

8 - ***B. ranunculoides*** L.

Group B - *'Trachycarpa'*

Annual herbs. Leaves herbaceous, subamplexicaul. Bracts present. Fruits papillose.

9 - *B. semicompositum* L.

10 - *B. tenuissimum* L.

Group C - 'NW African origin'

Shrubs, subshrubs or perennial herbs. Leaves herbaceous or coriaceous, subamplexicaul. Bracts present. Fruits smooth.

11 - *B. acutifolium* Boiss.

12 - *B. album* Maire

13 - *B. balansae* Boiss. & Reut.

14 - *B. barceloi* Coss. ex Willk.

15 - *B. benoistii* Litard. & Maire

16 - *B. canescens* Schousb.

17 - *B. dumosum* Coss. & Balansa

18 - *B. fruticescens* L.

18a - subsp. *fruticescens*

18b - subsp. *spinosum* (Gouan) O.Bolòs & Vigo

19 - *B. lateriflorum* Coss. ex H.Wolff

20 - *B. montanum* Coss.

21 - *B. oligactis* Boiss.

22 - *B. plantagineum* Desf.

23 - *B. salicifolium* R.Br. ex Buch

24 - *B. subspinosum* Maire

II) Subgenus *Penninervia* S.S.Neves *subgenus nov.*

Shrubs or perennial herbs. Leaves pinnate-reticulate, with thick midrib and a reticulum of fine lateral veins, or more rarely ± parallel-veined, with or without secondary veins, but all veins thick and very prominent, especially the intramarginal vein.

Type of subgenus: *B. fruticosum* L.

Group D - *'Penninervia'*

Shrubs or perennial herbs. Leaves coriaceous or herbaceous, pinnate-reticulate, with thick midrib and slender reticulate lateral veins. Fruits with narrowly winged ridges.

25 - *B. angulosum* L.

26 - *B. foliosum* Salzm. ex DC.

27 - *B. fruticosum* L.

28 - *B. gibraltarium* Lam.

Group E - *'Crassinervia'*

Perennial herbs. Leaves coriaceous, ± parallel-veined, all veins very prominent. Fruits with filiform ridges.

29 - *B. rigidum* L.

29a - subsp. *rigidum*

29b - subsp. *paniculatum* (Brot.) H.Wolff

10.5 Key to identification of species of *Bupleurum*

1a - Upper leaves perfoliate. Bracts absent. **2**

1b - Upper leaves subsessile or ± amplexicaul, more rarely with a short petiole, not perfoliate. Bracts present, rarely deciduous (falling during fruiting), in which case bracts scars visible. **3**

2a - Fruit surface tuberculate (tubercles visible on the ovary after anthesis). Upper leaves ovate-lanceolate to ovate, or elliptic. ..... **1. *B. lancifolium***

2b - Fruit (ovary) surface smooth. Upper leaves ovate to suborbicular. ..... **2. *B. rotundifolium***

3a - Leaves pinnate-reticulate, with thick midrib and slender reticulate lateral veins. Shrubs, subshrubs or perennial herbs. [subgenus *Penninervia*]. **4**

3b - Leaves ± parallel-veined, sometimes with secondary veins. Shrubs, subshrubs, perennial or annual herbs. [subgenus *Bupleurum*, except *B. rigidum*] **7**

4a - Perennial herb, woody stock at the base, stems herbaceous. Basal leaves ± crowded, present during flowering. Cauline leaves cordate-amplexicaul. Bracteoles suborbicular to oblong, erect-patent. ..... **25. *B. angulosum***

4b - Shrub or subshrub, stems becoming woody. Basal leaves withering before flowering. Bracteoles ovate to lanceolate, reflexed or erect-patent. **5**

5a - Leaves oblong-lanceolate to oblong-elliptic, more rarely obovate, green upper surface (darker), glaucous underneath. Umbels terminal (only exceptionally with lateral umbels). Bracts deciduous during fruiting. ..... **27. *B. fruticosum***

5b - Leaves linear-lanceolate to oblong-lanceolate, upper and lower surfaces with similar colour (usually glaucous). Umbels terminal and lateral, the latter always present. Bracts persistent during fruiting. **6**

6a - Umbels with 1-3(-4) rays. Bracts ovate, erect-patent. Lower leaves linear-lanceolate to narrowly oblong-lanceolate, acute to acuminate, rarely uncinate. ..... **26. *B. foliosum***

6b - Umbels with (3-)5-25(-57) rays. Bracts ovate to lanceolate, reflexed. Lower leaves narrow- to broadly oblong-lanceolate, acute, mucronate-uncinate. ..... **28. *B. gibraltarium***

7a - Lower leaves with clearly prominent veins, thick intramarginal vein present and of similar prominence as midrib, sometimes with thick secondary veins. Basal leaves very long, (10-)15-45 cm long, sometimes attenuating into petiole. Perennial herb, leaves coriaceous. ..... **29. *B. rigidum***

7b - Lower leaves with visible veins, sometimes slightly raised, but without thick intramarginal vein, sometimes with slender secondary veins. Basal leaves (3-)5-15(-20) cm long, rarely attenuating into petiole. Shrub, subshrub or perennial/annual herb, leaves coriaceous or herbaceous **8**

8a - Annual herb, stem and leaves herbaceous. Fruits smooth or papillose. **9**

8b - Perennial plant, woody at least at the base, stems woody or herbaceous, leaves coriaceous or herbaceous. Fruits smooth. **14**

9a - Bracts and bracteoles ovate to lanceolate, bracteoles (1-)1.5-5 mm broad, much broader than flowers or fruits. Fruits smooth. **10**

9b - Bracts and bracteoles linear to linear-lanceolate, bracteoles 0.3-1.5 mm, narrower than or of similar width as flowers and fruits. Fruits smooth or papillose. **11**

10a - Bracteoles ± translucent between veins, clearly showing 3 main veins connected by ascending veinlets (often abruptly recurved); bracteoles often spreading. Flowers or fruits easily seen, very unequally pedicellate, pedicels 1-8 mm long. ..... **6. *B. odontites***

10b - Bracteoles surface opaque, veins visible, veinlets often visible but not recurved; bracteoles generally erect-connivent (coming closer together). Flowers or fruits generally enveloped by the bracteoles, pedicels subequal and short, 1-3.5(-5) mm long. ..... **3. *B. baldense***

11a - Fruits (ovary) papillose, ovoid or subglobose, 1-2(-2.5) mm long. **12**

11b - Fruits (ovary) smooth, oblong to oblong-elliptic, 2-6(-7) mm long. **13**

12a - Fruits 1-1.5(-2) mm long, with whitish papillae, ridges inconspicuous (not visible). Umbels terminal and lateral, with peduncles of similar length. Bracteoles much longer than flowers or fruits which are very unequally pedicellate. ..... **9. *B. semicompositum***

12b - Fruits 1.2-2.5 mm long, with brownish papillae, ridges (generally) clearly visible and crenulate. Umbels terminal and lateral, the latter (generally) shortly pedunculate, almost axillary. Bracteoles often short and little longer than flowers or fruits, rarely much longer; pedicels short and subequal. ..... **10. *B. tenuissimum***

13a - Umbels with 1-4(-7) rays, 0.6-3.3 cm long, subequal, sometimes unequal. Bracteoles shorter than or of similar length to fruits, sometimes longer. Fruits 4-6(-7) mm long. Leaves 0.2-0.8 cm broad. ..... **7. *B. praealtum***

13b - Umbels with (1-)3-8(-10) rays, 0.2-6 cm long, generally very unequal. Bracteoles generally longer than fruits. Fruits 2-3.5 mm long. Leaves 0.2-0.4 cm broad. ..... **5. *B. gerardii***

14a - Umbels very compact, rays very short, generally hidden by the sessile flowers (rays sometimes longer in terminal umbels). Lateral umbels sessile. Petals with inflexed apex fimbriate. (*Morocco*). .... **12. *B. album***

14b - Umbels and umbellules clearly pedunculate, rays always clearly visible, flowers sessile or ± pedicellate. Lateral umbels short or long pedunculate. Petals with inflexed apex entire to 2-lobed. **15**

15a - Perennial herb, woody only at base, stems herbaceous. Leaves herbaceous (soft to the touch). **16**

15b - Perennial plant, generally a shrub or subshrub, with woody stems. Leaves coriaceous (± rigid) or herbaceous. **23**

16a - Basal leaves present during flowering (verify whether the very base of specimen is present). **17**

16b - Basal leaves withering before flowering (follow this lead if only upper stems are available). **19**

17a - Basal leaves few, abruptly attenuate into ± long petiole, withering during fruiting. Fruits narrowly winged. ..... **4. *B. falcatum***

17b - Basal leaves crowded, caespitose (growing in tufts or patches), sessile, gradually attenuate to the base, persistent during flowering and fruiting. Fruits with filiform to narrowly winged ridges. **18**

18a - Basal leaves (2-)5-13(-18) cm long, linear to oblong-lanceolate. Bracteoles 2-8 x 1-3 mm, ovate to lanceolate, longer and broader than flowers or fruits. Flowers (4-)6-25 per umbellule. Fruits with filiform ridges. (*Europe*). ..... **8. *B. ranunculoides***

18b - Basal leaves 1-2(-3.5) cm long, lanceolate to oblong-lanceolate. Bracteoles 1-2 x 0.3-0.5 mm, linear to lanceolate, shorter and narrower than flowers or fruits. Flowers 3-12 per umbellule. Fruits narrowly winged. (*Morocco*). ..... **15. *B. benoistii***

19a - Fruits narrowly winged, ridges appear as narrow ± scarious bands, lighter coloured than fruit surface. **20**

19b - Fruits with filiform or prominent ridges of similar colour as fruit surface. (follow this lead if fruits are not available). **21**

20a - Bracteoles 2-5(-8) x 0.8-1.5 mm, longer and broader or of similar size as fruits. Fruits 2-4 mm long. Cauline leaves sometimes falcate (curved like a sickle). (*Europe*). ..... **4. *B. falcatum***

20b - Bracteoles 1.5-4 x 0.3-0.5 mm, generally narrower and shorter than fruits. Fruits (4-)5-6(-7) mm. Cauline leaves not curved (*NW Africa*). ..... **20. *B. montanum***

21a - Leaves (0.1-)0.3-1.3 cm broad, obtuse to acute, sometimes mucronate. Petals with darker (but never blackish) mid-vein. Fruits narrowly winged. (*Morocco*). ..... **20. *B. montanum***

21b – Leaves 0.1-0.6 cm broad, acuminate. Petals sometimes with darker mid vein, brownish or blackish. Fruits with filiform to narrowly winged ridges. (*Iberian Peninsula / Balearic Islands*). **22**

22a - Rays (3-)5-12, thin but ± rigid. Petals with blackish mid-vein or spot. Fruits narrowly winged. (*Balearic Islands*). ..... **14. *B. barceloi***

22b - Rays 3-9, slender and flexible. Petals with or without darker, light brownish, mid-vein. Fruits with filiform ridges (*Iberian Peninsula*). ..... **11. *B. acutifolium***

23a - All lateral umbels 1-rayed, terminal with 1-3 rays. Rays 0.2-0.6(-1.5) cm long. Flowers 1-3(-4) per umbellule. (*Morocco*). .... **24. *B. subspinosum***

23b - Lateral umbels with 1 to more rays, but at least some with 2 or more rays. Rays 0.4-5 cm long. Flowers (1-)3-15(-20) per umbellule. **24**

24a - Rays thick, stiff, tapering towards the tip, spinescent (looking like spines after falling of fruits). ..... **18. *B. fruticescens* (subsp. *spinosum*)**

24b - Rays slender or thick, ± cylindric, not spinescent. **25**

25a - Leaves herbaceous, 0.5-2.5(-3.5) cm long, in clusters (= bundles), often clearly spaced along the stems, only upper nodes single-leaved; veins visible, but not raised. Rays generally subequal, slender. Flowers & fruits subsessile or shortly pedicellate, pedicels 0.5-1.5 mm long. ..... **17. *B. dumosum***

25b - Leaves herbaceous or coriaceous, 0.5-15(-18.5) cm long, sparse or sometimes crowded, but not in clusters; veins visible, sometimes raised. Flowers & fruits sessile or pedicellate, pedicels 0.3-8.5 mm long. **26**

26a - Lateral umbels shortly pedunculate, much smaller than terminal umbel (one lateral umbel per upper node). Leaves obovate to oblong-lanceolate, narrow marginal band minutely serrulate. ..... **19. *B. lateriflorum***

26b - Lateral umbels often long-pedunculate, similar or smaller than terminal umbels. Leaves linear to lanceolate, or oblong-lanceolate, narrow marginal band smooth or minutely serrulate. **27**

27a - Leaves oblong to oblong-lanceolate, sometimes obovate, obtuse, mucronate-uncinate (with a short hooked mucro). Shrub 100-200 cm tall. (*S Morocco & Canary Islands*). ..... **16. *B. canescens***

27b - Leaves linear to lanceolate or oblong-lanceolate, acute to acuminate, tips straight or uncinate (very rarely obtuse and then with a short straight mucro). Shrub, subshrub or perennial herb, (10-)15-200 cm tall. **28**

28a - Leaves coriaceous, lower leaves broadly lanceolate to oblong-lanceolate, 2-3(-4.2) cm broad, flat, acute to acuminate, rarely obtuse, with short straight mucro. (*Algeria*). ..... **22. *B. plantagineum***

28b - Leaves herbaceous or coriaceous, lower leaves linear to lanceolate, 0.1-2.2 cm broad (when more than 1.5 cm broad always herbaceous), acute to acuminate, with straight or uncinate tips. **29**

29a - Flowers and fruits sessile or subsessile, pedicels 0.3-1 mm long. Leaves ± coriaceous, 0.1-0.3(-0.5) cm broad, linear to linear-lanceolate, acuminate, with straight tips; veins clearly visible, slightly raised on both surfaces. (*NW Africa*). ..... **13. *B. balansae***

29b - Flowers pedicellate, pedicels (1-)2-10 mm long. Leaves coriaceous or herbaceous, 0.05-3(-4.2) broad, linear to lanceolate, or oblong-lanceolate, apex obtuse to acuminate, straight or uncinate; veins visible, sometimes slightly raised beneath, rarely on both surfaces. **30**

30a - Leaf apex generally uncinate. Flowering stems rigid, becoming woody. **31**

30b - Leaf apex ± straight. Flowering stems herbaceous, withering after flowering. **32**

31a - Leaves ± coriaceous, 0.05-1 cm broad, linear to oblong-lanceolate, often concave, sometimes subulate, gradually tapering towards the apex, generally uncinate (tips abruptly curving back). Bracteoles linear, 0.2-0.5 mm broad. (*Iberian Peninsula*). ..... **18. *B. fruticescens***

31b - Leaves herbaceous, sometimes coriaceous, 0.3-1.8(-2.2) cm broad, linear-lanceolate to broadly lanceolate, generally flat, generally abruptly narrowing at the often uncinate apex. Bracteoles linear-lanceolate to lanceolate, sometimes ovate-lanceolate, 0.3-1.2 mm broad. (*Madeira & Canary Islands*). ..... **23. *B. salicifolium***

32b - Leaves obtuse to acute, herbaceous, ± flat. Pedicels of flowers generally short, 1-4.5(-7) mm long. Fruits narrowly winged. ..... **20. *B. montanum***

32a - Leaves acuminate, herbaceous or coriaceous, ± flat or involute. Pedicels of flowers and fruit often long, (2-)3-10 mm long. Fruits with filiform or narrowly winged ridges. **33**

33a - Petals with very dark (blackish) mid-vein or spot. Fruits narrowly winged. Rays (3-)5-12, thin but ± rigid. (*Balearic Islands*). ..... **14. *B. barceloi***

33b - Petals with or without darker mid-vein (light brown, never blackish). Fruits with filiform ridges. Rays 1-9, slender and flexible. **34**

34a - Leaves coriaceous, ± flat and straight. Narrow marginal band generally minutely serrulate, at least in the lower leaves. (*Morocco*). ..... **21. *B. oligactis***

34b - Leaves herbaceous or coriaceous, flat or involute, straight or curved. Narrow marginal band smooth. (*Iberian Peninsula*). .. **11. *B. acutifolium***

10.6 Species descriptions

This section includes all the information gathered for each of the 29 species delimited in the present work . The order of species follows that given in the synopsis of the classification (section 10.4).

See section 10.2 for explanation on the organisation of this species account, and also for general information on references, typification and the maps of distribution.

1) *Bupleurum lancifolium* Hornem., *Enum. pl. hort. hafn. Supplement.* 2: 3 (1809).

Type: Lectotype (designated here) – Herb. Hornem.: "These plants ha[ve] been cultivated in Hort. Bot. in Copenhagen from seeds received from Paris in 1803. To Paris came these seeds from Egypt with the label: '*Bupleurum* d'Egypte Nectoux O.S. sr Ch.'. O. Hagerup." (C!).

Type locality: "Missum ex horto paris, sub nomina *Bupl.* d'Ægypte."

Synonyms: *Bupleurum intermedium* Poiret in Lam., *Encycl. Suppl.* 5: 585 (1817); *B. subovatum* Link ex Spreng., *Sp. Umbell.*: 19 (1818); *B. protractum* Hoffmans. & Link, *Fl. portug.* 2: 387 (1820); *B. heterophyllum* Link, *Enum. hort. berol. alt.* 1: 262 (1821).

Name origin: From the Latin '*lanci-*' (= lanceolate) and '*folium*' (= leaf), because of its lanceolate leaves.

Illustrations: Fiori & Paol., *Iconogr. fl. ital.* (3 ed.): 274, fig. 2232' [as 'v. *longifolium* Desf.'] (1933); Tutin, *Umbellifers Brit. Isl.*: 115 (1980); Valdés *et al.* (eds), *Fl. Andalucía Occid.* 2: 309 (1987); Jafri in *Fl. Libya*, Jafri & A. El-Gadi (eds), 117: 63, fig. 20A-F (1985); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 440 (1990).

Annual herb 6-75 cm tall, stems herbaceous, little branched. *Leaves* herbaceous, obtuse (very rarely acute or acuminate), mucronate, narrow marginal band smooth, sometimes minutely crenulate, ± parallel-veined, with 6-30 main veins, visible, radiating from the base, with delicate secondary veins, thick intramarginal vein absent; *basal leaves* (3-)5-15 x 1.5-6 cm, amplexicaul, gradually attenuate to the base, oblong to ovate-lanceolate, withering before flowering; *upper leaves* different in shape and insertion to lower, 1.5-6 x 1-3 cm, perfoliate, ovate-lanceolate to ovate, sometimes elliptic. *Umbels* terminal and lateral, all similar or the lateral smaller; *rays* 2-3(-4), 0.5-3 cm long, subequal or unequal, slender. *Bracts* absent. *Bracteoles* (4-)5(-6), 2-16 x 3-15 mm, subequal or unequal, ovate to ovate-lanceolate, acute to obtuse, mucronate, much longer and broader than flowers or fruits. *Flowers* 6-25 per umbellule; *petals* yellow, generally without darker mid-vein, inflexed apical lobe

entire. *Fruits* shortly pedicellate, pedicels 1-4 mm long; mericarps 3-5 x 1-2 mm, oblong to oblong-elliptic, tuberculate; ridges filiform and smooth.

Chromosome Numbers: 2n = 16 (Cauwet, 1979a).

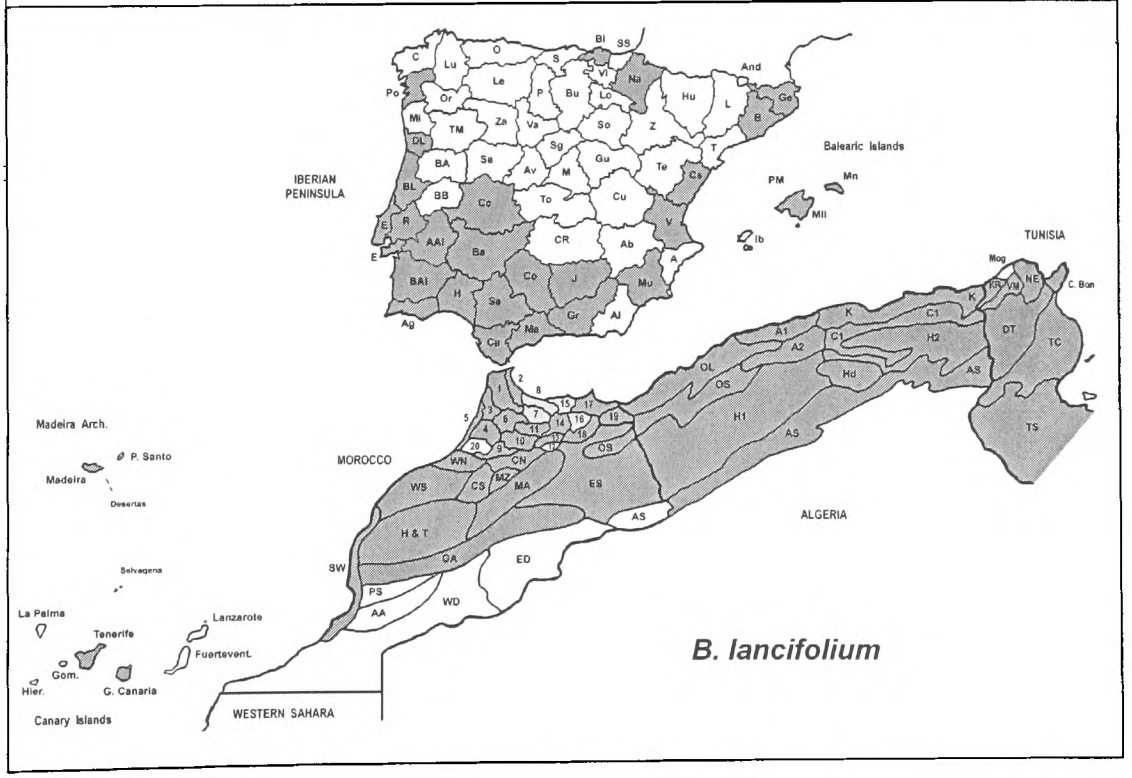
Ecology: Pasture, cereal fields, cultivated land and its margins, casual in gardens, road sides, railway banks, in disturbed soil; on clay, lime, and calcareous or chalky dry soils. *Secalion mediterraneum*, *Secalion cerealis*.

Altitude: 0-1500 m. **Flowering time:** (Mar.-)Apr. – Aug.

World Distribution: S Europe, N Africa & SW Asia. Introduced in Macaronesia, and also in Britain.

W Mediterranean & Macaronesian Distribution:

Spain: B, Ba, Bi, Ca, Cc, Co, Cs, Ge, Gr, H, J, Ma, Mu, Na, PM [Mll, Mn], Po, Se, V. *Portugal*: AAl, Ag, BAl, BL, DL, E, R. *Morocco*: 1, 2, 3, 4, 5, 6, 7?, 8?, 9, 10, 11, 12?, 13, 14, 15?, 16?, 17, 18, 19. CN, CS, ES, GA, H & T, OS, MA, MZ, SW, WN, WS. *Algeria*: A1, A2, AS, C1, H1, H2, Hd, K, OL, OS. *Tunisia*: C. Bon, DT, KR, NE, TC, TS. *Macaronesia* (Introduced): Madeira Arch. [Mad., P. Sto?]; Canary Isl. [G. Can., Tener.].



Notes: This species is indicated for all N Morocco (Jahandiez & Maire, 1932, p. 529-530), but I have not seen herbarium material from the regions 7, 8, 12, 15 & 16. There is one citation (19th century) of *B. lancifolium* for the island of S. Miguel (Azores), but the species has not been found since (Palhinha, 1966), and so it is apparently extinct in the archipelago. In any case, the species today is generally regarded as an introduced weed in Macaronesia – see also comments of Pitard & Proust (1909) under '*B. protractum*'.

Vernacular names: bou redim (Arabic); niella (Catalán); perfolhada (Portuguese); coleta, garravereta, garroveta, leóntica, perfoliada (Spanish).

Representative specimens:

Spain: Balearic Islands, Mallorca, Cúber, 24.v.1953, Palau Ferrer (MA 161319); Barcelona, Anoia, 14.vi.1985, Nuet Badia (BC 674923); Cáceres, Navalmoral de la Mata, 29.iv.1984, Ruis Téllez (SALAF 10223); Granada, Moraleda de Zafayona, 19.iv.1980, Ladero *et al.* (MA 340360); Jaén, Cazorla, "arroyo" (stream) of the Monte Sión, 2.vi.1983, A.M. Hernández (MA 340361); Vizcaya, Santurtzi, 31.v.1985, I. Zorrakin (MA 478815). **Portugal:** Algarve, Porches, 5.v.1977, A. Matos & L. Cabral 14105 (COI); Baixo Alentejo, Beja, Ervidel, 2.v.1962, M. Silva 2556 (LISE); Beira Litoral, Cantanhede, 28.v.1980, A. Marques 1965 (COI); Estremadura: Oeiras, c. Cruz Quebrada, 16.v.1957, A. Teles & M. Silva 445 (LISE). **Morocco: 3** - Tetouan, c. Ksar el Kebir, 20.v.1985, C. Blanchén *et al.* (MA 299403); **10** - Fès, c. Sidi Harazem, 24.v.1981, Fernández Casas 4974 (MA 435765). **Algeria: OL** - c. Oran, 1.vii.1933, A. Faure (MA 86148); **K** - W of Tizi-Ouzou, Draa-Ben-Kheda, 29.iv.1976, D.A & S.J. Sutton 977 (RNG). **Tunisia: KR** - Souk-el-Arba, v.1909, C.J. Pitard (MA 86143); **TS** - Gafsa, iii.1908, C. J. Pitard (RNG). **Macaronesia:** Madeira, "In arvis prope Gorgulho", iv-v.1865-1866, G. Mandon 122 (COI - Herb. Willk.; E).

Conservation status:

This species has a fairly broad distribution, but in many places has been introduced mixed with other seeds (especially cereals). Nevertheless, the species seems in decline in many areas, probably as a consequence of the replacement or abandonment of traditional crops and farming techniques.

Critical taxonomic notes:

1) The protologue cited here for *Bupleurum lancifolium* Hornem. is an earlier publication than the one cited in the literature [*Hort. bot. hafn.*: 267 (1813)].

2) After recently seeing the type material of *B. lancifolium* Hornem. I finally understood why some authors (e.g. Snogerup, 1972) prefer to divide the material I regard here as *B. lancifolium* into two different species. The type material of *B. lancifolium* (originally from N Africa – "*Bupl.* d'Ægypte") consists of very small

plants that are not common in European material. Snogerup (1972) considered that the larger plants should be classified as a different species: *B. intermedium*. Nevertheless, the differences indicated by Snogerup for these two taxa are rather continuous, and basically all based on size: the taller plants have larger leaves, larger inflorescences, larger fruits, longer styles, etc. However, there is a more relevant difference between these taxa: a slightly different shape of the cauline leaves (basal leaves wither quite early in development, and often it is not possible to know their shape). Cauline leaves attenuate (gradually) much more to the apex in the type material of *B. lancifolium* than in the most common material of the species – this is probably the reason why Hornemann described the leaves of his new species as '*foliis ovato-lanceolatis perforatis acuminatis*'. Snogerup (1972) noticed this character but did not seem to rely on it, as it was not used in the identification key (he used fruit dimensions to separate *B. lancifolium* and *B. intermedium*). In the Iberian material there are specimens with uncommon narrowly lanceolate leaves, but they do not attenuate as much to the apex as the type material [e.g. Spain: Granada, Moraleda de Zafayona, 19.iv.1980, Ladero *et al.* (MA 340360)]. I have seen a few N African specimens with similar leaf shape to the type material [e.g.: Tunisia: Sousse, iv.1907, C.J. Pitard 132 (MA 86141); Gafsa, iii.1908, C.J. Pitard 391 (MA 86142; & RNG)]. But, still, this difference seem only to merit varietal rank, and, if that is the case, 'var. *lancifolium*' would be much less common than 'var. *intermedium*'. This division has already been proposed, although with different nomenclature, by Pottier-Alapetite (1979) in her *Flore de la Tunisie*. She distinguished two varieties in *B. lancifolium*: 'var. *intermedium*' and 'var. *heterophyllum*' (the later would correspond to 'var. *lancifolium*'). Jafri (1985) in *Flora of Libya* described the peculiar different shape of the leaves of the typical *B. lancifolium* ("leaves usually with a tapering acumen" – see also Fig. 20A in this work), but also divided the material into two different species: *B. lancifolium* and *B. subovatum* (this is a later synonym, so in any case *B. intermedium* has priority).

3) *B. lancifolium* is often mistaken for *B. rotundifolium*, but these species are easily distinguished by the surface of the fruits, smooth in the latter and clearly tuberculate in the former. If a specimen is only in flower, tubercules can be seen on the surface of the ovary from an early stage of the flower development (the rough surface is noticeable after anthesis).

Typification notes:

The type specimen has two different labels, neither of which were placed by the author, Hornemann. The first label was written by O. Hagerup (dated 18/5/1949). The second label was written by I. Linczevski in 1949; here he indicated the specimen as the 'typus' – I have not found any evidence of publication of the type by this author. Attached to the specimen there is a packet with a few mericarps of the 'original collection' sent from Paris in 1803. However, the handwriting in the packet seems to be the same as that of O. Hagerup. On the back of the specimen, is written: '*Bupleurum lancifolium.*' 18/5 or 1815?, with a 'signature' that could be a 'H' [of Hornemann?]. Although all evidence seems *a posteriori*, I assume that this specimen can be considered original material because it belongs to Hornemann's herbarium, and therefore can be designated as lectotype.

There is another Hornemann specimen of *B. lancifolium* in C that, apparently, comes from the same cultivated material as the lectotype. It has written on the back: "*Bupleurum lancifolium* ex hort. Haun." [different handwriting than in the lectotype]. Linczevski labelled the specimen as 'isotypus'. However, it seems better to consider this specimen only as syntype, as it is not possible to prove that it is a duplicate of the chosen type.

2) *Bupleurum rotundifolium* L., *Sp. pl.*: 236 (1753).

Type: Lectotype – Herb. Linn. 335.1 (LINN!) [selected by Rechinger, K.H. & Snogerup in K.H. Rechinger (ed.), *Flora iranica* 162: 272 (1987)].

Type locality: "Habitat inter Europae australis segetes."

Synonym: *Bupleurum perfoliatum* Lam., *Fl. franç.* 3: 405 (1778).

Name origin: From the Latin '*rotundus*' (= almost circular) and '*folium*' (= leaf), because of the shape of the upper leaves.

Illustrations: Fiori & Paol., *Iconogr. fl. ital.* (3 ed.): 274, fig. 2232 (1933); Tutin, *Umbellifers Brit. Isl.*: 113 (1980); Valdés *et al.* (eds), *Fl. Andalucía Occid.* 2: 309 (1987); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 439 (1990).

Annual herb 10-70(-85) cm tall, stems herbaceous, little branched. *Leaves* herbaceous, obtuse, mucronate, narrow marginal band ± smooth, ± parallel-veined, with 6-30 main veins, visible, radiating from the base, with delicate secondary veins, thick intramarginal vein absent; *basal leaves* 4-10 x 2-5 cm, amplexicaul, gradually attenuate to the base, oblong to ovate-lanceolate, withering before flowering; *upper leaves* different in shape and insertion, 1-6 X 1-5 cm, perfoliate, ovate to suborbicular. *Umbels* terminal and lateral, all similar or the lateral sometimes smaller; *rays* (2-)3-8(-11), 0.3-2 cm long, subequal, sometimes unequal, slender. *Bracts* absent. *Bracteoles* 4-5(-6), 5-15 x 2.5-7(-14) mm, subequal or unequal, ovate to ovate-lanceolate, acute to obtuse, mucronate, much longer and broader than flowers or fruits. *Flowers* 5-15 per umbellule; *petals* yellow, generally without darker mid-vein, inflexed apical lobe entire. *Fruits* shortly pedicellate, pedicels 1.5-2 mm long; mericarps 2.5-4 x 1-1.5 mm, oblong to oblong-elliptic, smooth; ridges filiform and smooth.

Chromosome Numbers: 2n = 16 (Cauwet, 1979a).

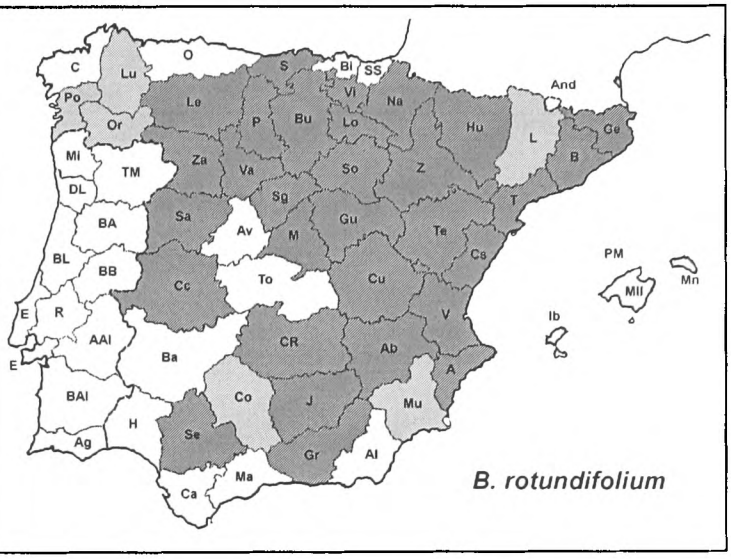
Ecology: Pasture, cereal fields and cultivated land, or in its margins; on lime or calcareous dry soils. *Secalietalia, Deschampsion mediae.*

Altitude: 0-2000 m. **Flowering time:** May – Jul.(-Aug.).

World Distribution: W, C & S Europe and SW Asia. Introduced in Britain, but now scarce. Also recorded in Algeria, but probably introduced.

Distribution in the Iberian Peninsula:

Spain: A, Ab, B, Bu, Cc, (Co), CR, Cs, Cu, Ge, Gr, Gu, Hu, J, (L), Le, Lo, (Lu), M, (Mu), Na, (Or), P, (Po), S, Sa, Se, Sg, So, T, Te, V, Va, Vi, Z, Za.



Notes: No material was seen from some of the provinces cited in the literature: Córdoba [Alameda – *Anales Jard. Bot. Madrid* 14: 633 (1956)]; Lérida [Riu Mosoll de la Coma y Querols – *Acta Geobot. Barcinon.* 1: 60 (1964)]; Lugo [Vertientes del Sil – *Brotéria* 20: 125 (1922); Murcia [Sierra de Espuña – *Lazaroa* 6: 260 (1984); & Gómez *et al.*, 1996]; Orense [Villamartín de Valdeorras – *Anales Jard. Bot. Madrid* 41(2): 371 (1985)]; and Pontevedra [Pasaje de Camposancos – Merino (1905)].

Vernacular names: bec-de-lièvre, unflabou (Catalán); thorow-wax (English); langarica (Euskera); haloche, oreja de liebre, perfoliada (Spanish).

Representative specimens:

Spain: Alicante, Sierra Aitana, 10.vii.1960, A. Rigual (MA 369580); Albacete, Robledo, 19.vi.1984, J. Herranz (MA 326570); Barcelona, Rupit, 15.vi.1949, Losa & Montserrat (BCF 42079); Burgos, Condado de Trebiño, 25.vi.1989, G. Zúñiga & J. Alejandre (MA 486221); Cantabria, between Potes and Monastery of S. Toríbio, 6.viii.1971, S. Talavera & B. Valdés (SEV 11832); Cuenca, Los Perales, 2.viii.1975, F. Casas & G. Guardia (MA 429179); Granada, Sierra de Baza, 4.vii.1984, J. Torres *et al.* (GDA 29020); Guadalajara, Utande, valley of the river Badiel, 27.v.1995, V.J. Arán & M.J. Tohá (MA 558688); Huesca, Yebra de Basa-Sobas, 17.viii.1988, P. Montserrat (MA 515858); Jaén, Sierra Mágina, Aguadulce, 13.vi.1925, Cuatrecasas (MAF 53002); León, Huergas de Gordón, 14.vi.1991, A. Penas & M. Garzón (MAF 137492); Madrid, Pinilla del Valle, 15.vi.1980, Fernández Gonzáles (MAF 121289); Navarra, Petilla de Aragón, 24.vii.1988, I. Aizpuru *et al.* (MA 459834); Palencia, Cervera de Pisuerga, 14.vii.1974, S. Rivas & E. Valdés (MAF 92044); Salamanca, Matilla de los Caños del Rio, 29.vi.1984, J. Sánchez & R. Belda (SALA 36396); Tarragona, Ulldemolins, 31.v.1974, J. Molero (BC 614968).

Conservation status:

Apparently *B. rotundifolium* used to be a common field weed in many areas of its total distribution, but it seems now to be in severe decline. This might be a consequence of the use of new methods in agriculture.

Critical taxonomic notes:

Bupleurum rotundifolium is often confused with *B. lancifolium*, but easily distinguished because the latter has tuberculate fruits.

Typification notes:

The following is the original material for *B. rotundifolium*: Herb. Burser 16: 1 (UPS - IDC microfiche!); Herb. Clifford 104, *Bupleurum* 2, fol. a (BM); Herb. Clifford 104, *Bupleurum* 2, fol. b (BM); Herb. Clifford 104, *Bupleurum* 2[beta] (BM); Herb. Linn. 335.1 (LINN!).

Culinary and ornamental uses:

The tender leaves and roots of *B. rotundifolium* have occasionally been used as a vegetable in salads and vegetable stews, or to season meat (Núñez & Castro, 1991). The flowering branches are used in Britain for home decoration.

3) *Bupleurum baldense* Turra in *Giorn. Italia Sci. Nat.* 1: 120 (1764).

Type: Neotype (selected here) – "Italia. Prov. Verona. In pascuis subalpinis montis 'Baldo'; solo calc. 400-1000 m.s.m. 1894", G. Rigo s.n. [Herb. F.C. Crawford] (E!).

Type locality: "In summitate Montis *delli Masi* prope Montem *della Corona.*" ['Dei Vegetabile di Monte Baldo'].

Synonyms: *Bupleurum odontites* auct. non L., *Sp. pl.*: 237 (1753); *B. divaricatum* auct. non Lam., *Fl. franç.* 3: 410 (1778) [= *B. odontites* L.]; *B. aristatum* auct. non Bartl. in Rchb., *Iconogr. bot. pl. crit.* 2: 70 (1824); *B. opacum* Lange in Willk. & Lange, *Prodr. fl. hispan.* 3: 71 (1874).

Name origin: From the type locality, Monte Baldo, in the region of Vicenza, Italy.

Illustrations: Tutin, *Umbellifers Brit. Isl.*: 117 (1980); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 447 (1990).

Annual herb 2-30 cm tall, stems herbaceous, diffusely branched. *Leaves* 1-8 x 0.2-0.5(-1) cm, herbaceous, subamplexicaul, linear to oblong-lanceolate, gradually attenuate to the base, acute to acuminate, narrow marginal band smooth, parallel-veined, 3-5 veins, visible, sometimes with delicate secondary veins, thick intramarginal vein absent; *basal leaves* sometimes attenuate into petiole, normally withering before flowering; *cauline leaves* sessile. *Umbels* terminal and lateral, all similar; *rays* 2-5, 0.1-1.7(-2.5) cm long, unequal, slender. *Bracts* 3-5, ovate-lanceolate, erect-patent, at least one bract longer than the longest ray, persistent in fruit. *Bracteoles* 5, 5-14 x 1.5-5 mm, subequal, erect-connivent, ovate to ovate-lanceolate, apiculate or aristate, much longer and broader than flowers or fruits. *Flowers* 3-9 per umbellule; *petals* yellow, with or without darker mid-vein, inflexed apical lobe entire. *Fruits* shortly pedicellate, pedicels 1-3.5(-5) mm long; mericarps 2-2.5 x 0.5-1 mm, oblong-elliptic, smooth; ridges filiform and smooth.

Chromosome Numbers: 2n = 16 (Cauwet, 1979a).

Ecology: Pasture and grassland, maquis, dunes and cliffs by the sea, stony places,

open and dry areas; on calcareous, chalky, slaty and sandy soils; occasional in cultivated land. *Thero-Brackypodium distachion.*

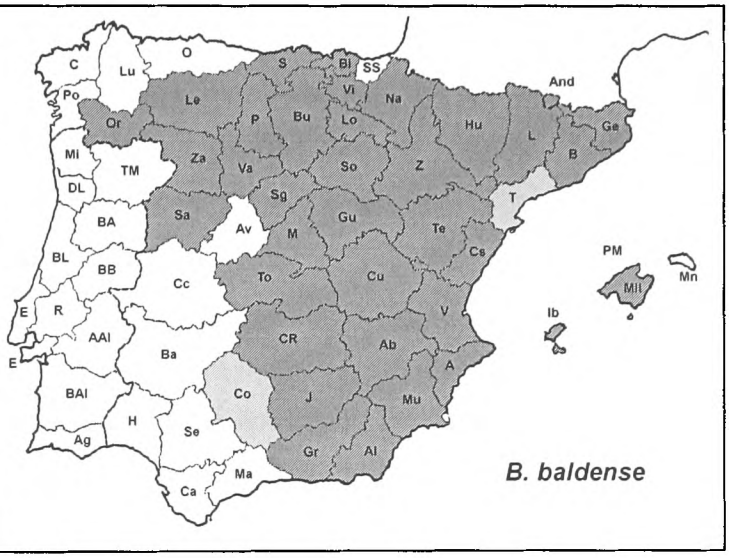
Altitude: 0-1600(-2200) m. **Flowering time:** Mar. – Jul.(-Aug.).

World Distribution: S & W Europe, from Spain & S England (very rare in the latter) to Romania, & Mediterranean islands.

Distribution in the Iberian Peninsula:

Andorra: And.

Spain: A, Ab, Al, B, Bi, Bu, (Co), CR, Cs, Cu, Ge, Gr, Gu, Hu, J, L, Le, Lo, M, Mu, Na, Or, P, PM [Mll, Mn, Ib & small islands], S, Sa, Sg, So, (T), Te, To, V, Va, Vi, Z, Za.



Notes: Although no material has been seen, there are citations of *B. baldense* for the provinces of Córdoba [Cabra, in Sierras Subbéticas – *Lagascalia* 16(1): 137 (1990)], and Tarragona (see Bòlos, 1998, p. 163).

Vernacular names: unflabou opac (Catalán).

Representative specimens:

Andorra: Mas d'Alins, Les Pardines, 4.vii.1992, G. Nieto Feliner (MA 513910). **Spain:** Alicante, V. Gallinera, 13.v.1995, J. Soller & Signes (MA 562798); Albacete, Cerrolloso, 28.vi.1988, A. Aparicio *et al.* (MA 504383); Almería, Valdeinfierno, vi.1990, A. Pall..[?] (MA 546027); Balearic Islands - Mallorca, Porto Cristo, Cuevas del Drach, 29.iv.1976, D. Sánchez-Mata (MAF 115523); Menorca, Ferreríes, 30.iv.1951, P. Montserrat (JACA 47851); Ibiza, Montes de San Juan, 15.iv.1949, Palau Ferrer (MA 86250); Barcelona, Moncada, El Turo, 9.vi.1976, S. Silvestre (SEV 38212); Burgos, Valle de Tobalina, 14.vi.1986, J. Alejandre (MA 364682); Ciudad Real, Quintos de Mora, 18.vi.1959, P. Montserrat (JACA 59259); Cuenca, Belinchón, 17.vi.1978, Pajarón (MA 310022); Granada, Sierra de Baza, Barranco del Relumbre, 10.vii.1984, J. Torres *et al.* (GDA 29018); Lérida, Cubells, 10.vi.1984, J. Pedrol (MA 418403); La Rioja, Quel, 11.vii.1992, G. Zúñiga & J. Alejandre (MA 533761); Orense, Rubiá, Vilar de Silva, 29.vi.1994, M. Carrrasco *et al.* (MA 543000); Teruel, Tortajada, 12.vi.1988, G. Mateo (MA 462997); Toledo, Ciruelos, 27.v.1981, P. Cantó *et al.* (MAF 109430); Valladolid, Esgueva, 13.vi.1981, J. Fernández Alonso (MA 338822); Zamora, Peñausente, 24.vi.1980, Sánchez Rodriguez (SALA 32690).

Conservation status:

The species is endangered in Britain, although not threatened in the rest of Europe (Wigginton, 1999). There are only two known localities in S England, one in the coast of Devon and the other in Sussex. Apparently, the species has been reduced by quarrying, urban development and change of land use, and also by excessive pressure of visitors to the coastal dunes, in particular during the summer.

Critical taxonomic notes:

1) *Bupleurum baldense* is easily recognizable because of its 'compacted' umbels. It has fairly broad bracts, longer than the rays, that partially cover the umbellules; also as the bracteoles are connivent, flowers and fruits are generally hidden within them.

2) *B. baldense* is sometimes mistaken for *B. semicompositum*, but the two species are easily distinguished by their fruits: smooth in the former and clearly papillose in the latter, which also has much narrower bracts and bracteoles.

3) Some Iberian material of *B. baldense* has been wrongly identified as *B. odontites* L. (q.v.) and *B. glumaceum* Sibth. & Sm., but, neither of these two taxa occur in the Peninsula. In any case, these taxa are easily differentiated by their ± translucent bracteoles, while in *B. baldense* they are always opaque (as the name of one of its synonyms suggests). *B. baldense* and *B. odontites* are also distinguishable by the pedicels of flowers/fruits (single umbellule): pedicels are subequal and short in the former, and very unequal (some of them quite long: up to 8 mm) in the latter.

Typification notes:

Turra's herbarium, formerly in the Museo Civico of Vicenza, was destroyed during the Second World War. There are still some of his specimens in LINN, but none for this particular taxon and a neotype is therefore required. The specimen selected here as neotype (E!) was collected from the type locality (Monte Baldo) and is in accordance with the original description and the present concept of the species. This specimen consists of 6 complete plants all in good condition. There is a duplicate at E (isoneotype), and it is likely that there are more duplicates in other herbaria.

4) *Bupleurum falcatum* L., *Sp. pl.*: 237 (1753).

Type: Lectotype – Herb. Burser 16: 10 (UPS - IDC microfiche!) [selected by Reduron, J.-P., in preparation].

Type locality: "Habitat in Misniae, Vallesiae sepibus." [Meissen, Germany; and Valais, Switzerland].

Synonym: *Bupleurum alpigenum* Jord. & Fourr., *Brev. pl. nov. fasc.* 1: 35-36 (1866).

Name origin: From the Latin '*falcatus*' (= curved like a sickle), because of its (sometimes) falcate leaves.

Illustrations: Jourd. & Fourr., *Icon. fl. Eur.* 2: tab. 270 (1870) [as *B. alpigenum*]; Fiori & Paol., *Iconogr. fl. ital.* (3 ed.): 275, fig. 2238 (1933); H.E. Hess *et al.*, *Fl. Schweiz* 2: 806 (1970); Tutin, *Umbellifers Brit. Isl.*: 121 (1980); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 444 (1990).

Perennial herb 20-80 cm tall, sometimes woody at the base, stems herbaceous, little branched, withering after flowering. *Leaves* 2-15(-18) x 0.3-2.5 cm, herbaceous, subamplexicaul, leaf blade linear-lanceolate to oblong, narrow marginal band smooth, parallel-veined, 3-6(-7) veins, with slender secondary veins, thick intramarginal vein absent; *basal leaves* differing in shape and insertion to upper leaves, petiolate, with blade abruptly attenuate into the petiole, obtuse to acute, withering during flowering, petiole up to 3/4 of the total length; *cauline leaves* sessile, acute to acuminate, sometimes falcate. *Umbels* terminal and lateral, all similar; *rays* 3-15, 0.3-3(-5) cm long, subequal or unequal, slender. *Bracts* 1-4(-5), linear, erect-patent, shorter than the rays, persistent in fruit. *Bracteoles* 5, 2-5(-8) x 0.8-1.5 mm, subequal, linear to lanceolate, acuminate, longer and broader or of similar size than flowers or fruits. *Flowers* (3-)5-15(-25) per umbellule; *petals* yellow, sometimes with darker mid-vein, inflexed apex entire or slightly 2-lobed. *Fruits* shortly pedicellate, pedicels 1-2(-3) mm long; mericarps 2-4 x 0.8-1.3 mm, oblong to oblong-elliptic, smooth; ridges narrowly winged and smooth.

Chromosome Numbers: 2n = 16. This is the most common chromosome number recorded for the European material. However, several others have been indicated for

the species: 14, 22, 26, 28, 32, etc. (see Cauwet, 1979a; Goldblatt, 1981-1988; and Goldblatt & Johnson, 1990-1998). Also, high aneuploidal variation has been registered in Asian populations (Ohta, 1991 & Li, 1994). Ohta (1991) recorded all possible numbers (2n) between 19 and 34, and also indicated counts of 12, 37 & 40. The high chromosome number variation seems to be another 'expression' of the same problem found in morphological studies: very high polymorphism.

Ecology: Pasture, woodland, sometimes heath, rocky places, in the open or in the shade; calcareous and clayey soils. Woods of *Corylus avellana*, *Fagus sylvatica*, *Fraxinus* spp., *Quercus* spp., *Pinus sylvestris* and *Pinus* spp. *Buxo-Quercetum pubescentis*, *Geranion sanguinei*.

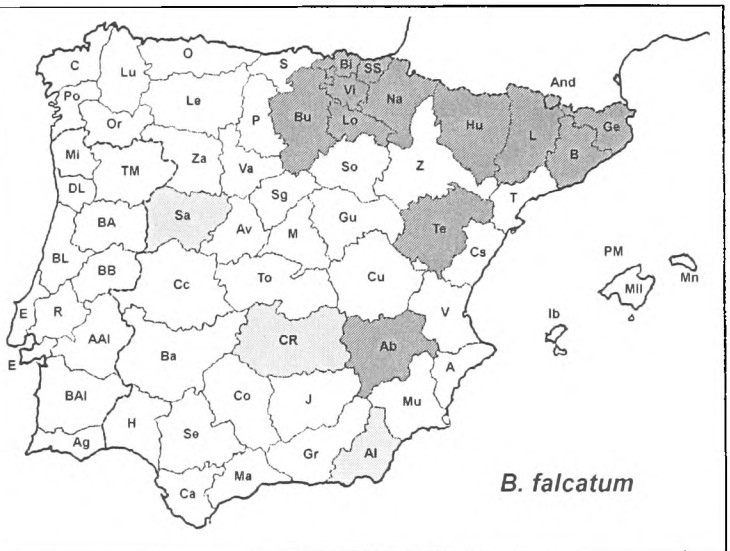
Altitude: 250-1900(-2200) m. **Flowering time:** Jun. – Aug.(-Oct.)

World Distribution: S, C & E Europe, and England (very rare); widespread in Asia (see Meusel, 1978).

Distribution in the Iberian Peninsula:

Andorra: And.

Spain: Ab, (Al), B, Bi, Bu, (CR), Ge, Hu, L, Lo, Na, (Sa), SS, Te, Vi.



<u>Notes</u>: *B. falcatum* occurs in Almería, Sierra de Gador, according to Sagredo (1987). The species is also cited for Salamanca, Santibáñez de Béjar, and Ciudad Real, Sierra Madrona [*Anales Jard. Bot. Madrid* 17(2): 369 (1959)]. The material cited for Salamanca is likely to be *B. praealtum*, as this species occurs in Béjar, and the two species are sometimes confused.

Vernacular names: ilebrenca, ilebrenca falcada (Catalán); sickle-leaved hare's-ear (English); hierba de la gitana, manzanilla de puerto, manzanilla fuerte (Spanish).

Representative specimens:

Andorra: Pont de la Margineda, border of river Valira, 4.vii.1992, C. Navarro *et al.* (MA 525659). **Spain:** Alava, Lagrón, Sierra de Cantabria, 15.viii.1992, J. Alejandre (MA 534084); Albacete, Calar del Mundo, vii.1991, J. Lara Ruiz (MGC 32931); Barcelona, Sierra del Cadi, entre Baga y Gosól, 1.viii.1926, Cuatrecasas (MAF 52879); Gerona, Camprodón, vii.[year?], M. Rivas Mateos (MAF 52877); Guipúzcoa, Otzaurte, 1.vii.1965, P. Montserrat (JACA 35765); Huesca - Panticosa, 4.viii.1979, Amich *et al.* (MA 310024); Torla, 23.vi.1989, Montserrat y Villar (MA 515855); La Rioja, Briones, 29.x.1925, Hno. Elias (MA 471119); Lérida, Bonaigüa, 22.vii.1975, E. Valdés *et al.* (MA 200443); Navarra, Goñi, 30.viii.1988, Uribe-Echebarria (MA 478717); Teruel, Puerto de Rudilla, 2.viii.1982, J. Ferrer (JACA 292384); Vizcaya - Abanto y Cierbana, 8.vi.1991, M. Zúñiga & J. Alejandre (MA 532932); Orozco, 30.viii.1986, J. Alejandre (MA 364747).

Conservation status:

Bupleurum falcatum is critically endangered in Britain, although not threatened in the rest of Europe (Wigginton, 1999). The species still survives, in very low numbers, in a single locality in Sussex, Norton Heath, and even this population was the result of re-introduction in 1988 (seeds from plants derived from the original population). The species has been reported in Britain as a casual in a few other localities. There is no record for Scotland, although I have seen a specimen of *B. falcatum* collected in a garden in Edinburgh – almost undoubtedly from 'imported' mixed seeds. Several factors were involved in the decline of the species in Britain; one of them is the short viability of the seeds (only about a year). This interesting detail might explain my almost total failure in germinating material of *B. falcatum* (see chapter 5).

Critical taxonomic notes:

1) The Iberian material of *B. falcatum* can all be included in a single taxon, without infraspecific subdivision, and corresponds to what is known as subsp. *falcatum* (see e.g. Tutin, 1968). However, in the rest of its area of distribution, *B. falcatum* is highly polymorphic, and numerous taxa (many at specific rank) have been described within this complex group (Davis, 1972). A complete revision of *B. falcatum* and associated taxa is greatly needed, but this is an intimidating task considering the large number of taxa in this group and, especially, the total area involved: from the Iberian Peninsula, throughout most of temperate Europe and Asia,

including also Japan (see Meusel, 1978, & Zoku, 1965).

2) *Bupleurum falcatum* can be confused with *B. ranunculoides*, but in the latter the basal leaves are sessile, linear to oblong-lanceolate, gradually attenuating to the base, and persistent during flowering and fruiting; also its bracteoles are normally broader. *B. falcatum* might also be mistaken for *B. praealtum*, especially when the basal leaves have withered. But *B. falcatum* has a larger number of rays and flowers, and its fruits are narrowly winged, with ridges appearing as narrow scarious bands, while in *B. praealtum* ridges are filiform and of the same colour than the rest of the fruit.

Typification notes:

The following is the original material of *B. falcatum*: Herb. Burser 16: 10 (UPS - IDC microfiche!); Herb. Linn. 116.7 (S - IDC microfiche!); [icon] in Cordus, *Annot. Diosc. Mat. Med.* 108 [non 69] (1561); [icon] in Rivinus, *Ordo pl. fl. pentapetal.*: 44 (1699).

Medicinal and other uses:

The leaves of *B. falcatum* have been occasionally used as a vegetable (Núñez & Castro, 1991). This species has been traditionally used as a medicinal plant in Asia, particularly in China, Japan, India and Pakistan (Baquar, 1989; Gorovoy *et al.*, 1980; Luo *et al.*, 1993a,b; Roi, 1955; and Satyavati *et al.*, 1976). In China and Japan, the dried roots of *B. falcatum*, and closely related species, are used for treatment of common colds with fever, malaria and typhoid fever (as an antipyretic), the feeling of tightness in the chest, hypochondriasis, and liver disorders. The active ingredients involved in the anti-inflammatory and antihepatotoxic effects are designated saikosaponins, a type of triterpene glycosides (Luo *et al.*, 1993b; Pistelli *et al.*, 1993). I have only found a reference of medicinal use of this species in Europe, for NE Spain (Villar *et al.*, 1987). Here, the infusion prepared with the flowering stems of *B. falcatum* is used to treat influenza and as an 'intestinal tonic' – the Spanish vernacular name 'manzanilla fuerte' suggests its use: 'manzanilla' = 'camomile' or infusion, 'fuerte' = strong.

5) *Bupleurum gerardii* All. in *Mélanges Philos. Math. Soc. Roy. Turin* [*Misc. Taur.*] 5: 81 (1774).

Type: Lectotype [icon!]: "*Bupleurum involucris et involucellis pentaphyllis, foliolis lineari-subulatis*" in Gérard, *Fl. gallo-prov.*: 234, fig. 9: (1761) [selected by Jafri in *Flora of Libya,* Jafri & A. El-Gadi (eds), 117: 69 (1985)].

Type locality: "Provenit in gallopr. australis sterilibus, campestribus." [in Gérard, *Fl. gallo-prov.* (1761) – the reference in the protologue].

Synonyms: *Bupleurum virgatum* Cav., *Descr. pl.*: 121 (1801); *B. filicaule* Brot., *Fl. lusit.* 1: 452 (1804); *B. jacquinianum* Jord., *Pugillus pl. nov. gallic.*: 71-72 (1852); *B. australe* Jord., *Pugillus pl. nov. galllic.*: 72-73 (1852).

Name origin: Dedicated to the French botanist Louis Gérard (1733-1819), who first described this taxon [*Fl. gallo-prov.*: 233-234 (1761)] but used polynomial nomenclature.

Illustrations: Gérard, *Fl. gallo-prov.*: 234, fig. 9 (1761); Rchb., *Iconogr. bot. pl. crit.* 2: tab. 164 & 165 (1824); Valdés *et al.* (eds), *Fl. Andalucía Occid.* 2: 310 (1987); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 450 (1990).

Annual herb 20-50(-70) cm tall, stems herbaceous, diffusely branched. *Leaves* all similar, 1-8 x 0.2-0.4 cm, herbaceous, subamplexicaul, linear to linear-lanceolate, slightly attenuate to the base, acuminate, narrow marginal band generally smooth, parallel-veined, 3-5(-8) veins, visible, without secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal and lateral, all similar, sometimes lateral smaller; *rays* (1-)3-8(-10), 0.2-6 cm long, generally very unequal, slender. *Bracts* 1-4(-6), linear, erect-patent, much shorter than the longest rays, persistent in fruit. *Bracteoles* 4-6, 4-9 x 0.5-1 mm, subequal, linear, acuminate, similar or much longer and of similar width than flowers or fruits. *Flowers* (1-)3-8 per umbellule; *petals* yellow, rarely with pinkish tones, sometimes with darker mid-vein, inflexed apical lobe entire. *Fruits* shortly pedicellate, pedicels 0.5-3 mm long; mericarps 2-3.5 x 0.5-1 mm, oblong to oblong-elliptic, smooth; ridges filiform and smooth.

Chromosome Numbers: 2n = 16 (Cauwet, 1979a).

Ecology: Mediterranean open vegetation and maquis, pasture, margins of lagoons, occasional on cultivated land; calcareous or clayey soils, rarely on slate, dry or subhumid soil. *Brachypodion phoenicoidis*, *Origanetalia*, *Agrostetalia*.

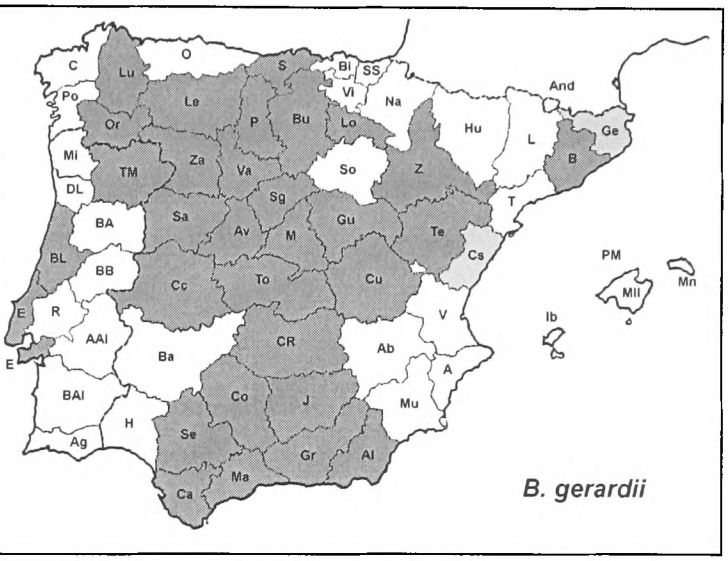
Altitude: 150-1400(-2000) m. **Flowering time:** Apr. – Jul.(-Aug.).

World Distribution: W & S Europe & SW Asia.

Distribution in the Iberian Peninsula:

Spain: Al, Av, B, Bu, Ca, Cc, Co, CR, (Cs), Cu, (Ge), Gr, Gu, J, Le, Lo, Lu, M, Ma, Or, P, S, Sa, Se, Sg, Te, To, Va, Z, Za.

Portugal: BL, E, TM.



Notes: No herbarium material of *B. gerardii* was seen for two of the Spanish provinces cited in the literature: Castellón [Castillo de Villamalefa – *Anales Jard. Bot. Madrid* 48(1): 78 (1990)] and Gerona [Alt Emporda – *Acta Phytotax. Barcinon.* 18: 77 (1976)].

Representative specimens:

Spain: Almería, Sierra de Gador, Fondón, 18.vi.1988, B. Valdés *et al.* (G); Ávila, Tremedal, 12.vii.1992, S. Sardinero (MAF 142019); Burgos, Ciruelos de Cervera, 11.vii.1979, Pons-Sorolla *et al.* (MA 413035); Cáceres, Serradilla, 16.vi.1981, Ladero & C. Valle (MA 310023); Ciudad Real, Sierra de S. Andrés, 14.vi.1988, D.S. Mata & J.E. Echebarría (MAF 127181); Guadalajara, Puebla de Beleña, 17.vi.1995, V.J. Aran & M. Tohá (MA 558668); León, Herreros, 21.v.1982, G. Blanca (GDA 14846); Madrid, Embalse de Santillana, 10.vii.1981, G. Navarro *et al.* (MA 310732); Orense, S. Justo de Valdeorras, 14.vi.1958, Bellot & Casaseca (MA 179168); Palencia, Monte Carrión, 24.vi.1950, M. Lainz (MA 155924); Salamanca, Linares de Rio, Pico Cervero, vii.1979, J.L.F. Alonso (MA 519175); Toledo, San Pablo, 7.vii.1977, A. Velasco (MAF 99677); Zaragoza, El Frasno, 23.vi.1990, A. Martinez (JACA 396690). **Portugal:** Beira Litoral - Coimbra, 30.vi.1980, A. Marques 2035 (COI); Fátima, Valinhos, 'Via Sacra', 26.vi.1994, S. Neves 1 (COI, E, MA); Estremadura, Sesimbra, 8.vi.1971, M. Béliz & J. Guerra (MA 325436); Trás-os-Montes, Bragança [cultivated in Coimbra], vi.1966, M. Queirós 2105 (COI).

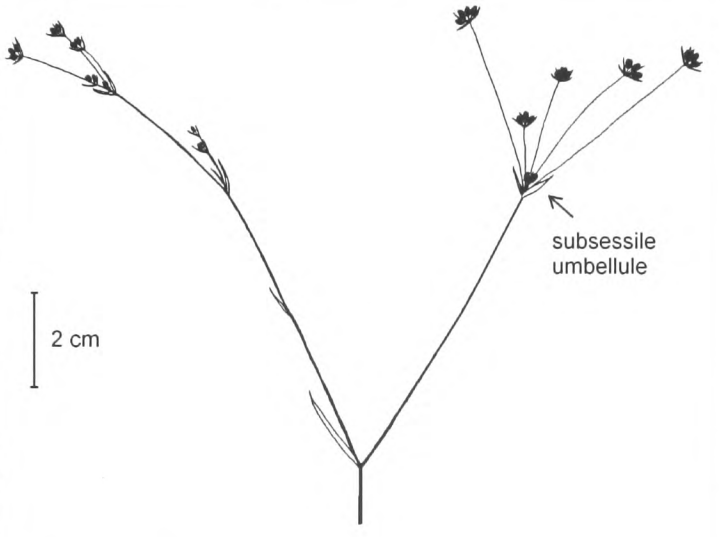
Critical taxonomic notes:

1) *Bupleurum gerardii* may be mistaken for its close relative *B. praealtum*. The only reliable morphological character to distinguish these two species is the size of mature fruits, quite often not available.

However, for most of the material there are other characters that help identification. *B. gerardii* flowers earlier (April-June), and is generally a smaller and more delicate plant, with umbels of several very unequal rays (some of them very long – up to 6 cm). *B. praealtum* flowers later (July-August), but is very variable in size (from c. 30 cm up to 1 m or more in height); its umbels normally have few (1-4, rarely 5-7), subequal rays (sometimes unequal) – see illustrations of *B. praealtum* on page 241. The relative length of the bracteoles/flowers has been used to distinguish the two taxa. In *B. gerardii*, bracteoles are often much longer than the flowers (or fruits), but some material has very short bracteoles (see Fig. A & B below). In *B. praealtum* in contrast, bracteoles are normally shorter or of similar size than flowers (or fruits), but again some specimens have very long bracteoles (see Fig. C of *B. praealtum*, p. 241). Length of bracteoles seems to be more characteristic of particular populations rather than the result of phenotypic plasticity. Next page drawings show the variation in bracteole length that can be found in *B. gerardii* – compare also with illustrations of *B. praealtum* (p. 241).

2) Ladero & Velasco (1978) subdivided the Iberian material of *B. gerardii* into 3 **subspecies**: 1) subsp. ***gerardii***; 2) subsp. ***filicaule*** (Brot.) P. Silva [wrongly referred in the text as 'subsp. *filicaule* (Brot.) P. Cout.'– Coutinho (1939) considered this taxon at species level; P. Silva published subsp. *filicaule* in *Agron. Lusit.* 30: 215 (1968)]; and 3) subsp. ***rouyana*** Ladero & Velasco [the latter subspecies as a new name based on the description of *B. gerardii* All. var. *affiniforme* Rouy & Camus, *Fl. France* 7: 334 (1901)]. These 3 taxa correspond to different populations of a single species, but the differences do not merit subspecific rank maybe only variety rank. Ladero & Velasco described *B. gerardii* subsp. *filicaule* as having 2-3 rays, plus "1 or 2 flowers in the base" of the umbels; however the material that corresponds to '*B. filicaule* Brot.' has quite often more than 3 rays (see Fig. A, showing a typical specimen of this taxon), and the 1-2 flowers in the base are more accurately described as subsessile umbellules, each with 1 flower and, at least, one bracteole,

the latter sometimes at the same level of the bracts. Fig. B illustrates the typical *B. gerardii* (see protologue & type), and would correspond to 'subsp. *gerardii*'. Therefore, the number of rays is not different in the three subspecies as suggested by Ladero & Velasco (1978, p. 506). Nevertheless, a more detailed study of material from the whole area of distribution of the species is needed to determine if there is good reason for infraspecific subdivision.



B. gerardii
short bracteoles

Fig. A - Portugal: Beira Litoral , Fátima , 26.vi.1994, S. Neves 1 (COI).

B. gerardii
long bracteoles

Fig. B - Spain: Granada, Lobras, 7.v.1980, J. Molero Mesa (MA 310947).

Typification notes:

1) Allioni provided a binomial name, *Bupleurum gerardii*, to the taxon described by Gérard in his *Flora gallo-provincialis* of 1761 (p. 233-234 – cited by Allioni as 'p. 23.', obviously a typographic mistake). The author also cited 'f. 9' [fig. 9] that corresponds to the illustration chosen by Jafri (1985) as the 'type' – the appropriate designation should be of lectotype. This illustration seems to be the only original material available. There could be a specimen of *B. gerardii* in Gérard's herbarium (TLON), but apparently most of this material is poorly annotated and without name of localities; in any case, such a specimen would only have been indirectly referred.

2) Original material of two of the synonyms of *B. gerardii* was studied during the visit to the Paris herbarium. Following is the typification of these two taxa:

Bupleurum australe Jord., *Pugillus pl. nov. gallic.*: 72-73 (1852).

Type: Lectotype (selected here) – "*Bupleurum australe* Jord. ined. *B. gerardii* [..?] non Jacq. Toulon. 1850." Jordan (Donné par Dr Grenier en 1875) (P!).

Type locality: "Hab. in incultis lapidosis siccis Galliae australioris; Toulon, Marseille, etc."

Bupleurum jacquinianum Jord., *Pugillus pl. nov. gallic.*: 70-71 (1852).

Type: Lectotype (selected here) – "*Bupleurum jacquinianum* Jord. *B. gerardii* Jacq. non Gérard. *B. gerardii* Godron Gr. en parte *B. affine* Boreau non Sadl. Lyon! Mezzienre. Jul. 49 [1849]." Jordan (P!).

Type locality: "Hab. in locis incultis siccis, ad agrorum margines, propè Lyon, ubi eum legi; ex Austriâ propè Vienne lectum et ex aliis locis accepi."

6) *Bupleurum odontites* L., *Sp. pl.*: 237 (1753).

Type: Lectotype – Herb. Linn. 335.11 (LINN!) [selected by Reduron, J.-P. in preparation].

Type locality: "Habitat in alpibus Vallesiae." [Canton of Valais, Switzerland].

Synonyms: *Bupleurum divaricatum* Lam., *Fl. franç.* 3: 410 (1778); *B. fontanessii* Guss. ex Caruel in Parl., *Fl. ital.* 8: 417 (1889).

Name origin: From the Greek '*odont-*' (= toothed). There is no obvious character of *B. odontites* that could resemble 'teeth'. Maybe Linnaeus wanted to describe the acuminate tips of the bracteoles. However, the 'toothed' umbellules may be easier to see in *B. baldense* (Linnaeus confused these two species – see taxonomic notes), because the bracteoles are connivent (they converge at the top).

Illustrations: '*Perfoliatum angustif. montanum*' in Colonna, *Minus cognit. rarior. ekphrasis*: 247 (1606); Rchb.f., *Iconogr. bot. pl. crit.* 2: tab. 177 (1824); H.Wolff in Engl., *Pflanzenr.* 43 (IV.228): 65, fig. 9R-T [as *B. fontanessii* Guss.] (1910); Pott.-Alap., *Fl. Tunisie* 1: 586, fig. 920 (1979); Jafri in *Fl. Libya*, Jafri & A. El-Gadi (eds), 117: 63, fig. 20G-K (1985).

Annual herb (5-)10-50 cm tall, stems herbaceous, ± branched. *Leaves* all similar, 2-12 x 0.15-0.5(-1) cm, herbaceous, subamplexicaul, linear to lanceolate, slightly or not attenuate to the base, acute to acuminate, narrow marginal band smooth or minutely denticulate, parallel-veined, 3-5(-7) veins, normally without secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal and lateral, all similar; *rays* 3-7, 0.3-1.7(-3.5) cm long, unequal, slender. *Bracts* 4-5(-6), lanceolate, erect to patent, longer than the longest rays, persistent in fruit. *Bracteoles* 5, 4-11(-20) x 1-2.5(-4) mm, subequal, often spreading, lanceolate to ovate-lanceolate, acute to acuminate, apiculate, translucent (between its veinlets), much longer and broader than flowers or fruits. *Flowers* 7-10(-13) per umbellule; *petals* yellow, with or without darker mid-vein, inflexed apex 2-lobed. *Fruits* with very unequal pedicels, 1-8 mm long; mericarps 1.3-1.8(-2.2) x 0.8-1 mm, oblong to oblong-elliptic, smooth; ridges filiform and smooth.

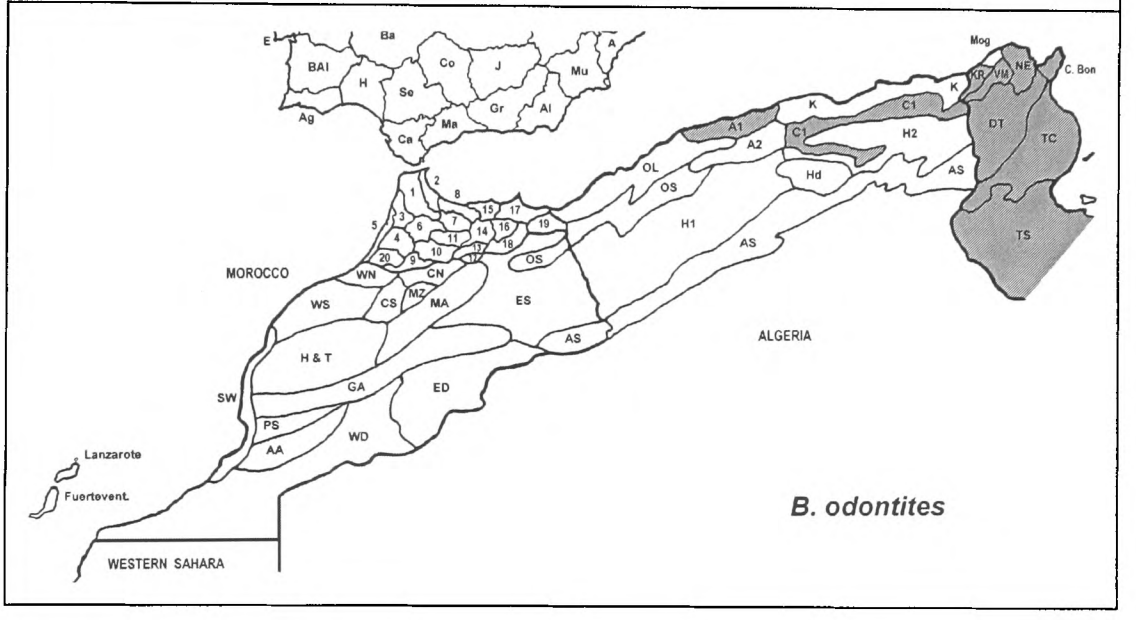
Chromosome Numbers: 2n = 16 (Cauwet, 1979a).

Ecology: Pastures, dry open habitats, stream banks, occasional as a weed and ruderal.

Altitude: 0-1300 m. **Flowering time:** (Mar.-)Apr. – Jul.

World Distribution: C, E & S Europe, eastwards to Palestine & Syria; N Africa.

Distribution in NW Africa: *Algeria*: A1, C1. *Tunisia*: C. Bon, DT, KR, NE, TC, TS, VM.



Representative specimens:

Algeria: A1 - Alger, "moissons [cultivated fiels] vers Mustafa", iv.1899, Pourzeille [?] (BC 25919). **C1** - Bouïra, "champs incultes près de la route d'Alger", vi.1905, J.A. Battandier (BC 25920; MA 425760). **Tunisia: C. Bon** – Hammam-el Lijar to Kroumbalia [Grombalia], iii.1883, E. Cosson *et al.* [Herb. J. Ball] (E). **DT** – 11 km from Grombalia on road to Ain Tebournouk. Zaghouan Province, 11.v.1990, P. Wilkin & E.J. Wellens 301 (RNG). **KR** – c. 14 km N from Jendouba, S of Tabarka, 11.v.1975, Davis & Lamond D57628 (E, RNG). **NE** - Djédeida, in segetibus, v.1909, C. J. Pitard (MA 86254).

Critical taxonomic notes:

1) This species is easily recognised by its very characteristic bracteoles: they are translucent between the veins – 3 main parallel veins, connected by transverse ascending veinlets, which are often abruptly recurved. In early development, the bracteoles are close together, but they soon enlarge and spread in different directions,

showing the very unequally pedicellate flowers (cf. with *B. baldense*).

2) Linnaeus confused two distinct species under the name *B. odontites*: **a)** this taxon in the sense used here (i.e. in the sense of *B. fontanessii* Guss. – see Tutin, 1968), and **b)** *B. baldense* Turra. The name has been used in both senses by different authors, e.g.: Boissier (1872, p. 839-840) and Fournier (1961, p. 669) used *B. odontites* in the sense of *B. fontanessii*; Rouy & Camus (1901, p. 336-337) used it in the sense of *B. baldense*; and Fiori (1925, p. 38-39) used it in both senses (he considered the 2 species as different varieties of *B. odontites*).

Tutin (1967) rejected *B. odontites* L. as a '*nomen ambiguum*', preferring *B. fontanessii* Guss. But even the latter name is not free of 'trouble': *B. fontanessi* Guss. was first published only as a *nomen* [*B. fontanessi* Guss., *Ind. Sem. Hort. Boccadifalco*: 3 (1825)]. Later, it was published by its own author, Gussone, as a synonym of *B. odontites* L. [*B. fontanessi* Guss., *Fl. sicul. prodr.*: 313 (1827) – *pro syn.*, *nom. inval.*: *Code* Art. 34.1(c)]. Eventually, many years later, the name was validly published by a different author, Caruel: *B. fontanessi* Guss. ex Caruel in Parl., *Fl. ital.* 8: 417 (1889). Tutin (1967) referred that the type locality cited by Linnaeus for *B. odontites* ("Hab. in alpibus Vallesiae" - Alps of Valais, South of Switzerland) could only have been a mistake. But the reference of this locality is likely due to the element '*B. baldense*' in Linnaeus concept of the species. *B. baldense* has been recorded in the southern Alps (Aeschimann & Burdet, 1989; Hess *et al.*, 1970), although not for the Canton of Valais, but in neighbouring areas (e.g. the type locality of *B. baldense* Turra is the 'Monte Baldo' in the Italian Alps). In this case, and considering the poor geographical precision of the earlier works, the citation of 'alpibus Vallesiae' is not so inaccurate.

Snogerup (1972) acknowledges that the name *B. odontites* 'has been mistreated', but still preferred to use *B. odontites* L. I do not see reason for further problems with the application of this name, as the lectotype chosen by Reduron (*in preparation*) corresponds only to material of *B. odontites* in its most generally accepted concept.

Typification notes:

The following is the original material of Linnaeus' *B. odontites*; note that it also includes material of *B. baldense* Turra: **a)** Herb. Burser 16.7 (UPS - IDC microfiche!) – a specimen of *B. baldense*; **b)** Herb. Linn. 335.11 (LINN!) - a

specimen of *B. odontites* **[lectotype]**; **c)** Herb. Linn. 335.12 (LINN!) - two specimens: on the right is *B. baldense*; on the left is *B. odontites*; **d)** [icon!] '*Perfoliatum angustif. montanum*' in Colonna (1606, p. 247) - undoubtedly an illustration of *B. odontites*.

7) *Bupleurum praealtum* L., *Amoen. acad.* 4 (70): 480 [*Fl. monsp.*] (1759).

Type: Neotype [icon!] – '*Bupleurum angustifolium*' in Dodoens, *Stirp. hist. pempt.*, 2 ed.: 633, left figure (1616) [selected by Snogerup in *Fl. Turkey*, P.H Davis (ed.), 4: 408 (1972)].

Type locality: "In lapidosis circa Mommau, la Colombiere & la Valette" [in Magnol, *Bot. Monsp.*: 42 (1686)] – localities in Montpellier, France.

Synonyms: *Bupleurum junceum* L., *Sp. pl.*, 2 ed., 1: 343 (1762).

Name origin: From the Latin '*prae*' (= very) and '*altum*' (= tall, high), because this species can grow up to 1 m or more of height (very tall if compared with the other herbaceous species of the genus).

Illustrations: Dodoens, *Stirp. hist. pempt.*: 633, fig. '*Bupleurum angustifolium*' (1616); Rchb., *Iconogr. bot. pl. crit.* 2: tab. 166 (1824); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 449 (1990).

Annual herb 30-100(-150) cm tall, stems herbaceous, little to much branched. *Leaves* all similar, 2-12(-20) x 0.2-0.8 cm, herbaceous, subamplexicaul, linear-lanceolate, slightly attenuate to the base, acuminate, narrow marginal band smooth or sometimes minutely serrulate, parallel-veined, 3-15 veins, visible, sometimes with prominent midrib beneath, without secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal and lateral, the latter smaller; *rays* 1-4(-7), 0.6-3.3 cm long, subequal, sometimes unequal, slender. *Bracts* 1-3, linear, erect-patent, shorter than the rays, persistent in fruit. *Bracteoles* 3-5, 1.5-3(-5) x 0.3-1 mm, subequal, linear, acuminate, shorter or sometimes longer and of

similar width to the flowers or fruits. *Flowers* 1-4(-6) per umbellule; *petals* yellow or yellow-greenish, generally without darker mid-vein, inflexed apical lobe entire. *Fruits* shortly pedicellate, pedicels 1-3.5 mm long; mericarps 4-6(-7) x 1-1.5 mm, oblong-elliptic, smooth; ridges filiform and smooth.

Chromosome Numbers: 2n = 16 (see *B. junceum* L. in Cauwet, 1979a).

Ecology: Pasture and woodland, in the open or in shade, dry and rocky places; calcareous or slaty soils. *Luzulo forsteri*, *Quercetum pyrenaicae*, *Brachypodion phoenicoidis*, *Cistion*, *Origanetalia.*

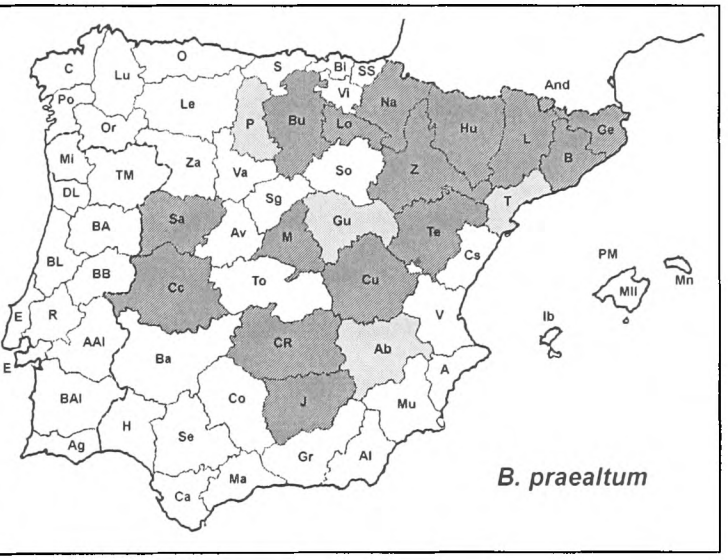
Altitude: 100-1200 m. **Flowering time:** Jul. – Sep.(-Oct.).

World Distribution: S & C Europe.

Distribution in the Iberian Peninsula:

Andorra: And.

Spain: Ab, B, Bu, Cc, CR, Cu, Ge, (Gu), Hu, J, L, Lo, M, Na, (P), Sa, (T), Te, Z.



Notes: No herbarium material was seen for some of the provinces cited in the literature: Albacete [Villarrobledo – *Lazaroa* 9: 231 (1986)]; Guadalajara [Cubillejo del Sítio – *Anales Jard. Bot. Madrid* 6(2): 62 (1945)]; Palencia [Villalaco – *Anales Jard. Bot. Madrid* 49(1): 126 (1991)]; and Tarragona (see Bolòs, 1998, p. 165).

Vernacular names: unflabou junci (Catalán).

Representative specimens:

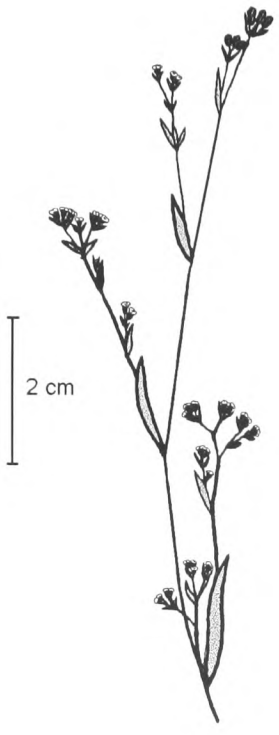
Andorra: Andorra La Vella, vii.1949, Losa & Montserrat (MA 152913); S. Julià de Lória, 4.vii.1992, P. Montserrat *et al.* (JACA 168992). **Spain:** Barcelona, Valvidrera, 1.x.1966, Rivas Martinez (MA 325246); Burgos, Oña, 12.ix.1983, P. Montserrat (MA 381948); Cáceres, Hervás, 10.viii.1946, Rivas-Goday (MA 179953); Ciudad Real, Ruidera, vii.1965, Borja *et al.* (MAF 69785); Cuenca, Hoz de Beteta, 3.ix.1966, R. Goday & Borja (MA 423411); Gerona, Ribes de Fresser, 28.vii.1967, J. Vigo (BC 146368); Jaén, Sierra de Cazorla, 31.vii.1983, A. Hernández (MA 541702); Lérida, Valle de Boi, Barruera, 23.viiii.1987, C. Aedo *et al.* (MA 449973); Huesca, Campo, 30.vii.1980, P. Montserrat & F. Fillat (JACA 2998880); Logroño (La Rioja), Ezkaray, peña S. Torcuato, 13.viii.1985, J. Alejandre (MA 333349); Madrid, El Boalo, 10.vii.1981, G. Navarro *et al.* (MAF 106808); Navarra, 26.viii.1988, P. Uribe-Echebarría (MA 478798); Salamanca, Montemayor del Rio, 26.vii.1982, F. Navarro & C. Valle (MA 292390); Teruel, Sierra de Albarracín, 10.vii.1965, E.F. Galiano *et al.* (SEV 38112); Zaragoza, Sigués, Las Tempranas, 20.viii.1990, J. A. Sesé (JACA 337393).

Critical taxonomic notes:

1) *Bupleurum praealtum* might be mistaken for *B. gerardii*, but the latter is normally a smaller and more delicate plant, with several rays, often very unequal, and with bracteoles (clearly) longer than flowers and fruits – see illustrations of *B. praealtum* (Fig. A, B & C) in page 241, and compare with *B. gerardii* (p. 233).

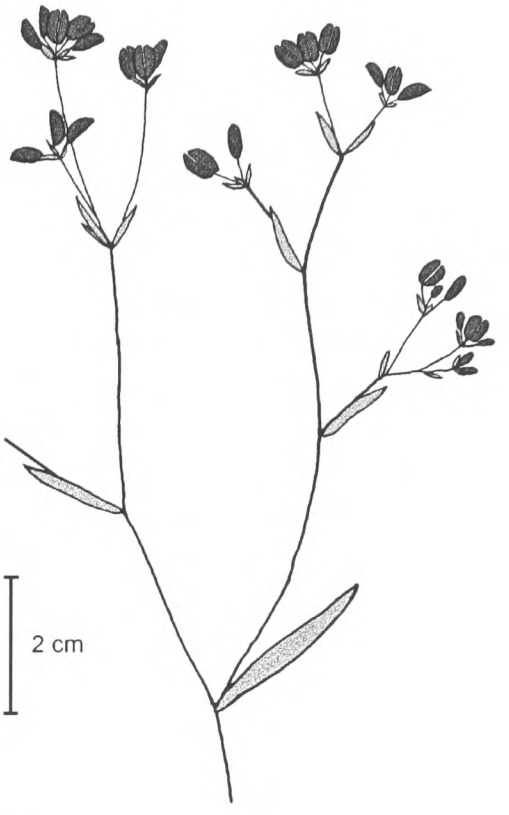
The specimen of *B. praealtum* in Fig. C (p. 241) has an unusual inflorescence, with very long bracts and bracteoles, but its height (c. 1 m tall), thick but hollow main stem, and leaves with fairly prominent midrib are typical of material of the species. ITS gene was sequenced for a specimen of the same population (Acc. No 308) as the one in Fig. C, and also for the specimen represented in Fig. A (Acc. No 289) – see chapter 9. The two specimens are apparently morphologically very distinct, but the molecular results confirm that they belong to the same species, *B. praealtum* (see chapter 9).

The specimen of Fig. C (MA 504255) could be mistaken for *B. falcatum*, specially because the basal leaves are no longer present, and so it is not possible to know if they attenuate into petiole; also some of its leaves are falcate (curved like a sickle) – *B. falcatum* is not the only species of *Bupleurum* that can have falcate leaves (they sometimes occur in other herbs of the genus). However, *B. falcatum* (q.v.) is a perennial herb with larger number of flowers (at least some umbellules with 10 or more flowers), and narrowly winged fruits (ridges appear as narrow scarious bands).



B. praealtum

Fig. A - Flowering specimen

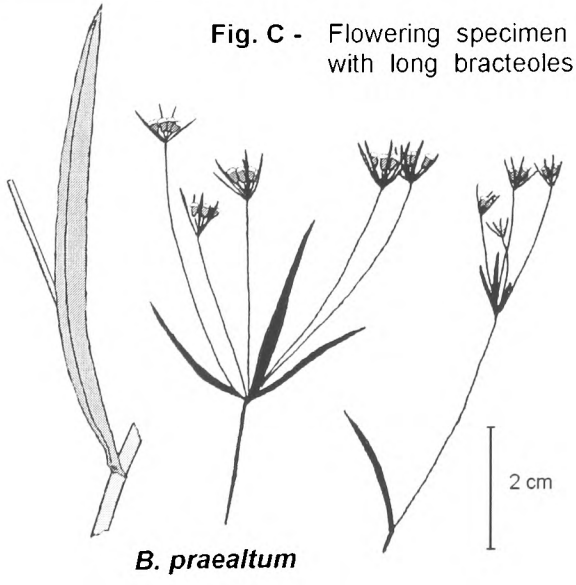


B. praealtum

Fig. B - Fruiting specimen

Fig. A - Spain: Teruel, Loscos, Piedrahita, 13.viii.1995, C. Fabregat & Lopez Udias (JACA 683295) - ITS gene was sequenced for this specimen (Acc. no 289).

Fig. B - Spain: Burgos, Oña, 12.ix.1983, P. Montserrat (G 294205).



B. praealtum

Fig. C - Flowering specimen with long bracteoles

Fig. C - Spain: Salamanca, Béjar, 6.vii.1988, G. Martin & G. Rowe (MA 504255).

Note:

ITS gene was sequenced for a specimen (Acc. no 308) of the same population and of similar morphology as the specimen shown in this figure.

2) Linnaeaus published for the first time the name *B. praealtum* (without description) in the first edition of its *Flora Monspeliensis* (1756). Here he provided a list of numbered binomial names for the polynomials used by Magnol in his *Botanicum Monspeliense* (1686 – Linnaeus copy was the 2 ed. of 1688, that is basically a reprint) – see Stearn (1973) for explanation of the link between Linnaeus' *Flora Monspeliensis* and Magnol's *Botanicum Monspeliense*. However, Linnaeus did not provide an explanation for the numbers listed under the 1756 edition of *Flora Monspeliensis*, and the names were published as *nomina nuda*. But, in the second edition of *Flora Monspeliensis* (1759 - published in the *Amoenitates Academicae*) he made clear the meaning of those numbers. Therefore, all the names listed became associated to a description in Magnol's *Botanicum Monspeliense*, and were validly published, although not the first time for most of them. *B. praealtum* is one of the names that was validly published for the first time in this second edition (1759) – see Stearn (1973, p. 635-637). The name *B. praealtum* is an earlier name for *B. junceum* L. (1762) – Linnaeus apparently preferred the latter (he did not use the name *B. praealtum* again), and was followed in his use by other authors, but the earlier name still has priority.

Typification notes:

The following is the typification of *Bupleurum junceum*, a synonym of *B. praealtum*. The original material of *B. junceum* includes what is now the type (neotype) of *B. praealtum*. I am chosing the same icon as the type of *B. junceum* to make the synonymy more obvious, so that the two names become homotypic.

Bupleurum junceum L., *Sp. pl.* 2 ed., 1: 343 (1762).

Type: Lectotype (selected here) – [icon!] – '*Bupleurum angustifolium*' in Dodoens, *Stirp. hist. pempt.*, 2 ed.: 633 ['474'], left figure (1616).

Type locality: "Habitat in Gallia, Italia."

Original material of *B. junceum* is as follows: Herb. Linn. (UPS); Herb. Linn. 335.24 (LINN!); [icon!] – '*Bupleurum angustifolium*' in Dodoens, *Stirp. hist. pempt.*: 633 ['474'], left figure (1616).

8) *Bupleurum ranunculoides* L., *Sp. pl.*: 237 (1753).

Types: Lectotype (selected here) – Herb. Burser 16: 8 (UPS - IDC microfiche!) [incomplete specimen]. Epitype (selected here) – Herb. Clifford 104, *Bupleurum* 3 (BM!).

Type locality: "Habitat in Helvetia, & Pyrenaeis."

Synonyms: *Bupleurum gramineum* Vill., *Prosp. Hist. pl. Dauphiné*: 23 (1779); *B. bourgaei* Boiss. & Reut. in Boiss., *Diagn. pl. orient.* Ser. 2, 3(2): 84-85 (1856); *B. ranunculoides* L. subsp. *gramineum* (Vill.) Hayek, *Prodr. fl. penins. balcan.* [*Feddes Repert. Spec. Nov. Regni Veg. Beih.* 30] 1: 971 (1927).

Name origin: Resembling a buttercup (*Ranunculus* sp.); because the umbellules are reminiscent of its flowers.

Illustrations: Rchb.f., *Icon. fl. germ. helv.* 21: tab. 1886, fig. II-III (1863); Fiori & Paol., *Iconogr. fl. ital.* (3 ed.): 275, fig. 2240 (1933); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 445 (1990).

Perennial herb (3-)10-30(-45) cm tall, stems herbaceous, little branched. *Leaves* 0.5-13(-18) x (0.1-)0.3-1.5 cm, herbaceous, ± amplexicaul, acute or acuminate, narrow marginal band smooth, parallel-veined, veins visible, generally without secondary veins, thick intramarginal vein absent; *basal leaves* crowded, not attenuate or gradually attenuate to the base, linear to oblong-lanceolate, 1-5 veins, persistent during flowering; *cauline leaves* sparse, sometimes different on shape, subamplexicaul, sometimes narrowly cordate-amplexicaul, linear to ovate-lanceolate, with 3 to numerous veins. *Umbels* terminal and lateral, all similar; *rays* (1-)3-6(-10), 0.25-4 cm long, subequal or unequal, slender. *Bracts* 1-3(-4), ovate-lanceolate, erect-patent, shorter than the rays, present during fruiting. *Bracteoles* 4-6, 2-8 x 1-3 mm, subequal, ovate to lanceolate, obtuse to acute, longer and broader than flowers or fruits. *Flowers* (4-)6-25 per umbellule; *petals* yellow, greenish or purplish, sometimes with darker mid-vein, inflexed apical lobe entire. *Fruits* short pedicellate, pedicels 0.5-2(-3) mm long; mericarps 2-4 x 0.8-1.5 mm, oblong to oblong-elliptic, smooth; ridges filiform and smooth.

Chromosome Numbers: 2n = 14, 21, 28 and 42 (Cauwet, 1979a).

Ecology: Pasture and open vegetation in high mountains, in shade or in the open, rocky places, generally on limestone, occasionally on conglomerate. Heath or moorland; *Poo-Festucetum sabinae*, *Pino-Juniperion sabinae*, *Seslerietalia coeruleae*.

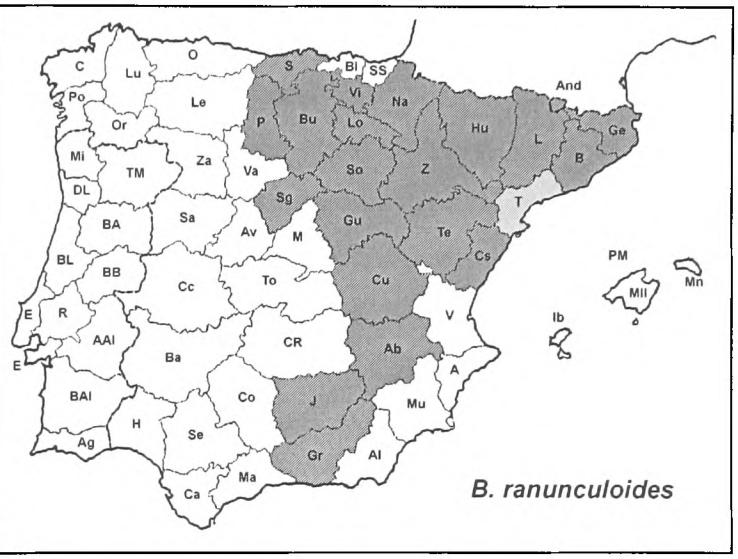
Altitude: (550-)800-2600 m. **Flowering time:** Jun. – Sep.

World Distribution: W, C & S Europe, from Spain to the Carpathians.

Distribution in the Iberian Peninsula:

Andorra: And.

Spain: Ab, B, Bu, Cs, Cu, Ge, Gr, Gu, Hu, J, L, Lo, Na, P, S, Sg, So, (T), Te, Vi & Z.



Notes: Citation of *B. ranunculoides* for the province of Tarragona (T) according to Bolòs (1998, p. 165).

Vernacular name: ilebrenca ranunculoide (Catalán).

Representative specimens:

Andorra: Puerto de Envalira, 8.viii.1983, E. Rico (GDA 24813). **Spain:** Albacete, Sierra de Alcaraz, 17.vii.1981, D. Sánchez-Mata (MAF 110151); Barcelona, Alt Berguedà – La Clusa, 3.viii.1976, A. Rosell (BC 621572); Burgos, Niela, 2.viii.1990, M. Gil Zúñiga & J. Alejandre (MA 493698); Cantabria, Aliva, Cueto de los Toribios, 17.viii.1950, E. Guinea (MA 165116); Castellón, cim de Penyagolosa [Peñagolosa], 15.vii.1967, J. Vigo (BC 146672); Huesca, Torla, Cotatuero, 20.viii.1987, P. Montserrat *et al.* (MA 515856); Jaén, Pontones, Sierra de Banderillas, 10.viii.1982, C. Soriano (MA 462383 – as *B. bourgaei* Boiss & Reut.); La Rioja (Logroño), Luezas, Serrezuela, 9.vii.1985, J. Alejandre (MA 333353); Lérida, Sierra del Cadi, 7.viii.1987, J. Pedrol & C. Pedrol (MA 495132); Navarra, Torralba del Rio, 7.viii.1988, P.M. Uribe-Echebarría (MA 484737); Palencia, Cervera de Pisuerga, 5.viii.1989, F. Betoño & J. Alejandre (MA 486227); Soria, Montenegro de Cameros, 1.viii.1991, J. Alejandre (MA 534400); Zaragoza, Añon, Macizo del Moncayo, 27.viii.1988, J. Alejandre (MA 486637).

Critical taxonomic notes:

1) All the Iberian material of *B. ranunculoides* L. can be included in a single taxon, without subdivision into subspecies. However, two subspecies have been recognised for European material: subsp. *ranunculoides* and subsp. *gramineum* (Vill.) Hayek (see Hayek, 1927; & Tutin, 1968). Subsp. *ranunculoides* refers to plants of larger size, with larger leaves and inflorescences, and, in particular, with cauline leaves often broadly cordate-amplexicaul, which never occur in Iberian material. The Iberian material would correspond only to subsp. *gramineum*, but more detailed study of the European material is required to ascertain if there is good reason for infraspecific subdivision.

The variation in size of leaves and inflorescences, and maybe total height, of the plants of *B. ranunculoides* might be partially due to the different levels of ploidy. This problem has been addressed before without definitive conclusions. At least one hexaploid population (2n = 42) of *B. ranunculoides* with exceptionally small plants (2-6 cm tall) has been identified (Küpfer, 1969), but the plants were growing under "very dry" conditions. Cauwet (1979b) investigated the relation between several characters and ploidy level, and did not find any correlation with macroscopic characters. Unfortunately, she only presented and discussed the results of fruit measurements (length), despite mentioning that several other morphological characters had been studied (height, relative dimensions of different organs, form of leaves, etc) – for some obscure reason these other characters did not have a "certain interest". However, at least the size of epidermal cells, namely stomata, seemed to show some correlation with ploidy level: stomata of polyploid plants (2n = 42) were clearly much larger than those of the diploid 'race' (2n = 14) – see Fig. 6 a-b, in Cauwet (1979b, p. 70).

2) *Bupleurum bourgaei* Boiss. & Reut. has up to now been considered an endemic of the Iberian Peninsula (Spain: prov. Albacete and Jaén) – see e.g. Rivas-Martínez *et al.*, 1991. However, this taxon does not show any particular distinguishing feature, and can be easily included within the range of variation of the populations of *B. ranunculoides*. Tutin (1968), although still treating this taxon as a separate species, noted that it was 'doubtfully distinct' from *B. ranunculoides* subsp. *gramineum*.

3) Material of *B. ranunculoides* has been wrongly identified as *B. angulosum* L., but the two species are easily distinguished by the venation type: parallel in the former and pinnate-reticulate in the latter; also *B. angulosum* has clearly winged fruits.

4) The species may also be confused with *B. falcatum* L. However, basal leaves of *B. falcatum* attenuate rapidly into a clear, ± long petiole (W European material), and wither during flowering; also its fruits are narrowly winged – ridges appear as narrow scarious bands.

Typification notes:

1) The following is the original material of *Bupleurum ranunculoides* L.: Herb. Burser 16: 8 (UPS - IDC microfiche!) - syntype (explicitly cited in the protologue); Herb. Clifford 104, *Bupleurum* 3 (BM!); Herb. Linn. 335.16 (LINN!) - both indirectly referred.

The specimen indicated here as lectotype is the only syntype, and was chosen in accordance with Art. 9.9 of the *Code*. However, this is an incomplete specimen, and to support the application of the name of the taxon an epitype was selected (Art. 9.7), which is also original material.

2) The following is the typification of *Bupleurum bourgaei,* here considered a synonym of *B. ranunculoides* (see taxonomic note 2 above):

Bupleurum bourgaei Boiss. & Reut. in Boiss., *Diagn. pl. orient.* Ser. 2, 3(2): 84-85 (1856).

Type: Lectotype (selected here) – Herb. Boiss.: "*Bupleurum paniculatum*, Brot. (Coss.). Sommet du Padron de Bien Servida", 22.vii.1850, E. Bourgeau (G-BOIS!). Syntypes in G-BOIS!

Type locality: "Hab. in cacumine montis *Padron de Bien Servida* in regno Granatensi orientali [Sierra de Alcaraz, prov. Albacete] cl. Bourgea[u] pl. exs. 1850 sub nomine *B. paniculati*".

9) *Bupleurum semicompositum* L., *Demonstr. pl.*: 7 (1753).

Type: Lectotype – Herb. Linn. 335.13 (LINN!) [selected by Townsend, C.C., Notes on the *Umbelliferae* of Iraq: III. *Kew Bull.* 20: 81-82 (1966)].

Type locality: "Habitat in Hispania."

Synonyms: *Bupleurum divaricatum* Lam. var. β., *Fl. franç.* 3: 410 (1778); *B. glaucum* Robill. & Cast. ex DC., Lam. & DC., *Fl. franç.* (3 ed.), 5: 515 (1815).

Name origin: From the Latin '*semi*' (= half) and '*compositus*' (= compound), because its compound umbels are reminescent of simple umbels.

Illustrations: Rchb., *Iconogr. bot. pl. crit.* 2: tab. 168 & 183 (1824); Fiori & Paol., *Iconogr. fl. ital.* (3 ed.): 275, fig. 2243 (1933); Valdés *et al.* (eds), *Fl. Andalucía Occid.* 2: 311 (1987); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 448 (1990).

Annual herb 2-35 cm tall, stems herbaceous, often much branched. *Leaves* 1-6 x 0.1-0.5(-0.8) cm, herbaceous, subamplexicaul, linear to linear-lanceolate, gradually attenuate to the base, acuminate, narrow marginal band smooth, parallel-veined, (1-)3-5 veins, visible, sometimes with delicate secondary veins, thick intramarginal vein absent; *basal leaves* sometimes attenuate into a short petiole, withering before or during flowering; *cauline leaves* sessile. *Umbels* terminal and lateral, all similar; *rays* 2-12, 0.1-2.5(-3) cm long, generally very unequal, sometimes with sessile umbellules, slender. *Bracts* 3-5, linear to linear-lanceolate, erect-patent to appressed, generally shorter than the longer rays, persistent in fruit. *Bracteoles* 5(-6), 2-8 x 0.5-1.5 mm, subequal, linear to linear-lanceolate, acuminate, sometimes apiculate, longer and broader than flowers or fruits. *Flowers* 2-9 per umbellule; *petals* yellow or greenish, sometimes purplish, with or without darker mid-vein, inflexed apical lobe entire. *Fruits* very unequally pedicellate, pedicels 0.5-6 mm long; mericarps 1-1.5(-2) x 0.4-0.7 mm, subglobose, papillose (whitish papillae); ridges inconspicuous.

Chromosome Numbers: 2n = 16 (Cauwet, 1979a).

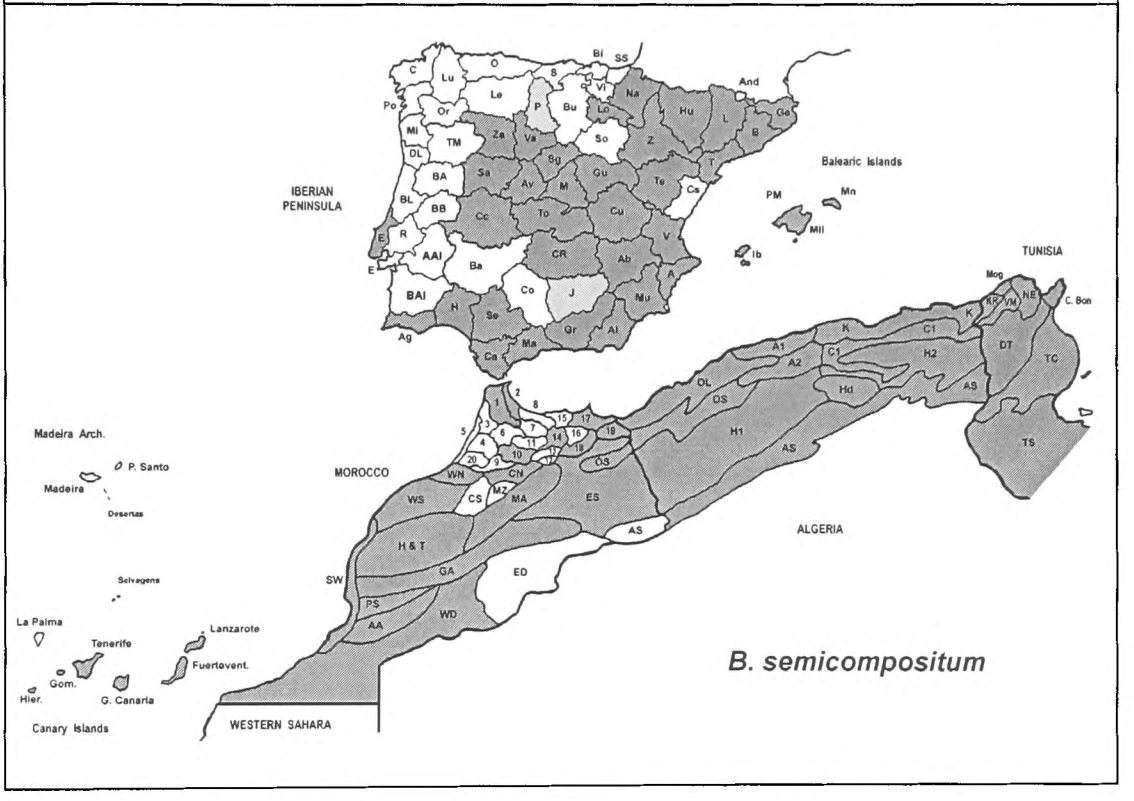
Ecology: Salt-marshes, pasture, margins of salty ponds or lagoons, calcareous, clayey, chalky and sandy dry soils. *Stipion capensis*, *Agropyro-Lygeion*.

Altitude: 0-500(-850) m. **Flowering time:** (Mar.-)Apr. – Jul.(-Sep.)

World Distribution: S Europe, N Africa, Canary Islands & SW Asia.

W Mediterranean Distribution:

Spain: A, Ab, Al, Av, B, Ca, Cc, CR, Cu, Ge, Gr, Gu, H, Hu, (J), L, Lo, M, Ma, Mu, Na, (P), PM [Mll, Mn, Ib & small islands], Sa, Se, Sg, T, Te, To, V, Va, Z, Za. *Portugal*: Ag, E. *Morocco*: 1, 2, 10, 14, 17, 18, 19. AA, CN, GA, H & T, MA, PS, SW, WD, WN, WS. *Algeria*: A1, A2, AS, C1, H1, H2, Hd, K, OL, OS. *Tunisia*: C. Bon, DT, KR, Mog, NE, TC, TS, VM. *Macaronesia*: Canary Isl. [Fuertev., G. Can., Gom., Hier., Lanz., Tener.]



Notes: Although no material has been seen, *B. semicompositum* is also cited from the Spanish provinces of Jaén [Pegalajar and Huesa – *Blancoana* 8: 45 (1990)] and Palencia [Dueñas – *Anales Jard. Bot. Madrid* 14: 468 (1955)].

Vernacular names: unflabou menut (Catalán); garrovereta (Spanish).

Representative specimens:

Spain: Albacete, Yeste, c. La Graya, 7.vi.1985, C. Soriano (MA 462382); Alicante, Salinas de Torre Vieja, 18.iv.1971, A.Rigual (MA 369584); Almería, Tabernas, 18.vi.1980, Diaz *et al.* (MA 461354); Balearic Islands, Ibiza, Bahía de S. Antonio, 1.v.1980, Rivas Martínez *et al.* (MA 422582); Cádiz, Trebujena, El Vento, 5.v.1989, A. Aparicio *et al.* (MA 468902); Ciudad Real, Daimiel, Isla del Morenillo, 12.v.1992, S. Cirujano (MA 552469); Cuenca, El Pedernoso, 18.v.1968, P. Montserrat (JACA 111268); Huelva, Almonte, El Martinazo, Doñana, 1.vi.1974, B. Cabezudo (SEV 18481);

Madrid, S. Fernando de Henares, 18.v.1983, M. Luceño (MA 371479); Navarra, Lodosa, 26.vi.1988, J. Alejandre (MA 466983); Salamanca, S. Cristóbal de la Cuesta, 29.vi.1987, F. Diez & J.S. Rodriguez (SALA 42276); Zamora, Tapioles, finca del Roble, 2.vi.1993, Ladero & C. Valle (SALA 90465). **Portugal:** Algarve, Cabo de S. Vicente, 21.iv.1981, Casaseca *et al.* (MA 310769); Estremadura, Sintra, S. Pedro, 27.v.1947, B. Rainha (MA 167324). **Morocco: 17** - Kebdana, El Garman, 27.iv.1933, Hno. Mauricio (BC 825987); **19** - Oujda, road to Taza, 29.v.1993, M. Etlaftski *et al.* (RNG); **AA** - 4 km SW of Ait Baha, 5.vi.1974, Reading Univ./BM Exped. 307 (E, RNG). **H & T** - Chemaia, 7.iv.1972, Davis 54155 (BM, E, RNG). **Algeria: H1** - 3 km S of Hassi Bahbah, 7.vi.1971, Davis 53271 (E). **Tunisia: TS** - Oudref, ii.1907, C.J. Pitard (E). **Canary Islands:** Fuerteventura, Bco. [Barranco] Grande near Betancouria, 9.v.1969, D. Bramwell 1611 (RNG). Gomera, iv.1908[?], M. Gandoger (COI). Lanzarote, "in rupestribus aridis supra oppidum Havia", iv.[19]24, O. Burchard 268 (E). Tenerife, Paso alto de Santa-Cruz, 22.iv.1855, H. de la Perradière (COI - Herb. Willk.; E).

Critical taxonomic notes:

1) *Bupleurum semicompositum* can be mistaken for *B. tenuissimum*, but the fruits easily distinguish these species: ridges are not visible in the former, while they are (generally) clearly marked in the second. But there are other characters that help identification, especially when fruits are not yet fully developed. *B. semicompositum* is divaricately branched, with numerous stems spreading in all directions; its umbels are all similar (lateral & terminal); bracts and bracteoles are always much longer than flowers or fruits, and fruits have whitish papillae. *B. tenuissimum* is normally diffusely branched, often with lateral umbels shortly pedunculate, almost axillary (rays of lateral umbels are also, in general, very short); bracteoles are often short and of similar length than fruits (rarely much longer); and fruits have brownish papillae.

2) Material of *B. baldense* has, on a few occasions, been wrongly identified as *B. semicompositum*. But these species are very easily distinguished by their fruits: always smooth in *B. baldense* and papillose in *B. semicompositum*. Also, bracts and bracteoles are much broader (ovate-lanceolate) in the latter species.

Typification notes:

The following is the typification of a synonym of *B. semicompositum*:

Bupleurum glaucum Robill. & Cast. ex DC., Lam. & DC., *Fl. franç.*, 3 ed., 5: 515 (1815).

Type: Lectotype (selected here) – "*Bupleurum glaucum* 'R. C.' Nice. Herbier de la Flora française (Bot. Gall.) donné au Muséum par A.P. De Candolle. 1822" (P!).

Type locality: "MM. Robillard et Castagne ont trouvé cette plante dans les lieux incultes, à Mazargue et la Gineste près Marseille; M. Requien, aux Sablettes près Toulon: je l'ai trouvée très-abondante sur les sables maritimes, à Nice".

10) *Bupleurum tenuissimum* L., *Sp. pl.*: 238 (1753).

Type: Lectotype – Herb. Linn. 116.13 (S - IDC microfiche!) [selected by Reduron, J.-P. in Jonsell & Jarvis, *Nordic J. Bot.* – in preparation].
Type locality: "Habitat in Germania, Anglia, Gallia, Italia."

Synonyms: *Bupleurum tenuifolium* L., *Amoen. Acad.* 4(70): 480 [*Fl. monsp.*] (1759); *B. procumbens* Desf., *Fl. atlant.* 1: 230 (1798); *B. columnae* Guss., *Fl. sicul. prodr., Supplementum*: 70 (1832).

Name origin: From the Latin '*tenuis*' (= slender, thin), because of its fine and delicate stems.

Illustrations: '*Bupleurum minimum*' in Colonna, *Minus cognit. rarior. ekphrasis*: 247 (1606); Desf., *Fl. atlant.* 1 [plates]: tab. 56 (1798); Rchb., *Iconogr. bot. pl. crit.* 2: tab. 167 (1824); H.Wolff in Engl., *Pflanzenr.* 43 (IV.228): 101, fig. 13 (1910); Fiori & Paol., *Iconogr. fl. ital.* (3 ed.): 275, fig. 2244 (1933); Tutin, *Umbellifers Brit. Isl.*: 119 (1980); Valdés *et al.* (eds), *Fl. Andalucía Occid.* 2: 310 (1987); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 449 (1990).

10) *Bupleurum tenuissimum* (Cont.)

Annual herb (4-)10-70 cm tall/long, stems herbaceous, diffusely branched. *Leaves* all similar, 0.5-8 x 0.05-0.6 cm, herbaceous, subamplexicaul, linear to linear-lanceolate, not attenuate or slightly attenuate to the base, acute to acuminate, narrow marginal band smooth, parallel-veined, 1-3(-5) veins, visible, sometimes with delicate secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal and lateral, the latter often with very short peduncles; *rays* 1-3(-6), 0.1-2 cm long, normally unequal, slender. *Bracts* 1-5, linear to linear-lanceolate, appressed or erect-patent, shorter or similar in size to the rays, persistent during in fruit. *Bracteoles* 5, 1-4(-6) x 0.3-1 mm, subequal, linear, acuminate, shorter to longer and narrower or of similar width than flowers or fruits. *Flowers* 1-6 per umbellule; *petals* yellow, greenish or purplish, sometimes with darker mid-vein, inflexed apical lobe entire. *Fruits* shortly pedicellate, pedicels 0.5-2 mm long; mericarps 1.2-2.5 x 0.8-1.2 mm, ovoid or subglobose, papillose (brownish papillae); ridges (generally) visible and crenulate.

Chromosome Numbers: 2n = 16 (Cauwet, 1979a).

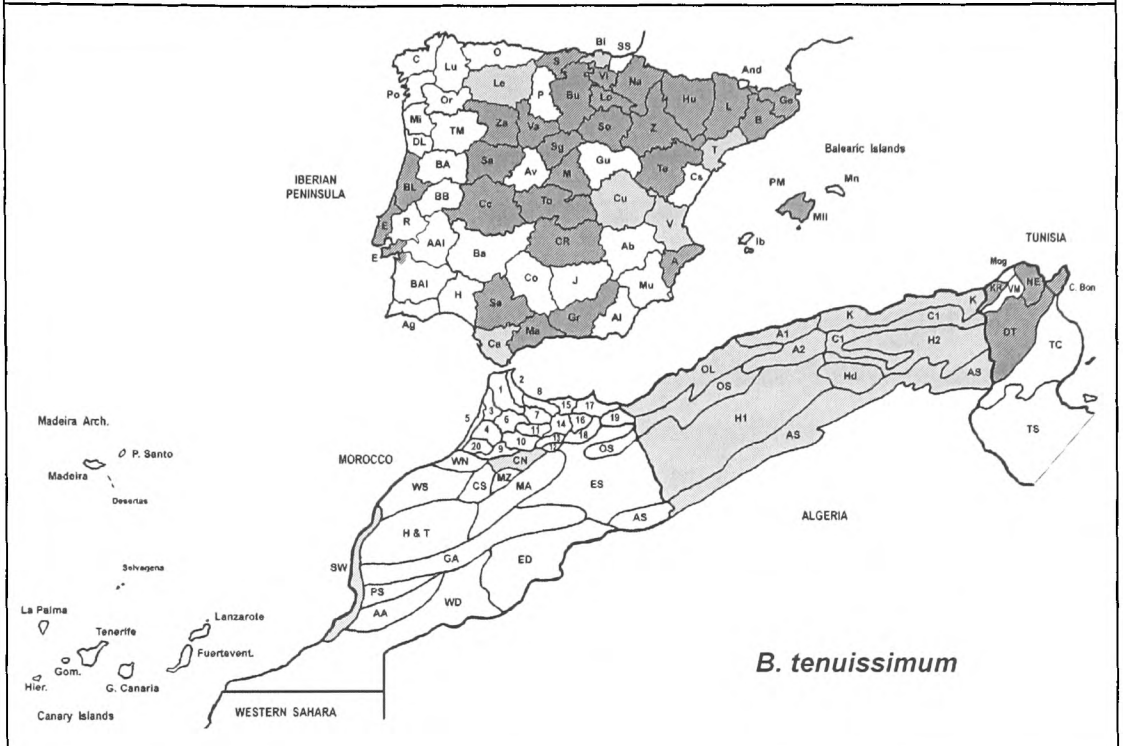
Ecology: Salt-marshes, margins of ponds, lagoons, streams or ditches; clayey and salty dry soils. *Aeluropo-juncetum subulati*, *Bupleuro-Juncetum gerardii*, *Holoschoenetalia*, *Phragmitetalia*, *Juncetalia maritimi.*

Altitude: 0-600(-1100) m. **Flowering time:** (Jun.-)Jul. – Oct.(-Nov.)

World Distribution: W, C & S Europe, S Britain and North Sea coasts, extending northwards to Sweden; NW Africa & SW Asia.

W Mediterranean Distribution:

Spain: A, B, (Bi), Bu, (Ca), Cc, CR, (Cu), GE, Gr, Hu, L, (Le), Lo, M, Ma, Na, PM [Mll], S, Sa, Se, Sg, So, (T), Te, To, (V), Va, Vi, Z, Za. *Portugal*: BAl, BL, E. *Morocco*: CN?, SW?. *Algeria* (rare): A1, A2, AS, C1, H1, H2, Hd, K, OL, OS. *Tunisia*: C. Bon, DT, KR, NE.



Notes: No material of *B. tenuissimum* was seen for some Spanish provinces cited in the literature: Cádiz [*Trab. Dept. Bot. Univ. Granada* 5: 11 (1978)]; Cuenca [Salinas del Manzano – *Anales Jard. Bot. Madrid* 38(1): 219 (1981)]; León [Quintanilla de Florez, and Andorcino – *Lazaroa* 8: 392 (1985); Tarragona (Bolòs, 1998, p. 166); Valencia [Sueca – *Anales Jard. Bot. Madrid* 36: 56 (1979)]; and Vizcaya (Bi) [Portugalete – *Lazaroa* 8: 392 (1985)].

Vernacular names: unflabou tenuíssim (Catalán); slender hare's ear (English); hinojillo de conejo, hinojillo de monte (Spanish).

Representative specimens:

Spain: Balearic Islands, Mallorca, Prat, 15.viii.1952, Palau Ferrer (MA 155930); Barcelona, Manleu, 14.xi.1925, Hno. Gonzalo (MA 86373); Burgos, Ayuelas, 12.x.1925, Hno. Elias (MA 86369); Cáceres, Sierra Gata, vii.[year?], Rivas Mateos (MA 53041); Cantabria, Argoños, 21.ix.1986, M. Herrera (MA 393306); Ciudad Real, Daimiel, Isla de Algeciras, 21.vii.1992, S. Cirujano (MA

552213); Gerona, S. Pere Pescador to Roses, 27.ix.1981, Molero y Rovira (BCF 42106); Granada, Cortijo de S. Andrés, c. Cullar-Baza, 1.xi.1977, J. Varo *et al.* (GDA 4011); Logroño, Anguciana, 15.ix.1986, Uribe-Echebarría (MA 478715); Navarra, Viana-Oyón, Las Cañas, 22.ix.1984, G. Montserrat & Uribe-Echebarría (MA 478796); Soria, Medinaceli, 5.x.1985, P. Montserrat & J. Vigo (MA 489086); Zamora, Molacillos, Arrañeras, 5.ix.1987, R. Garcia Rios (SALA 54312). **Portugal:** Baixo Alentejo, Alcácer do Sal, 18.ix.1950, F. Fontes & B. Rainha 4128 (COI); Beira Litoral, Vila Verde, c. Figueira da Foz, 16.ix.1994, S. Neves 22 (COI, E, MA). **Tunisia: NE** - bords du lac de Tunis à Mégrine, 11.x.1949, L. Faurel (BC 814979).

Critical taxonomic notes:

1) *Bupleurum tenuissimum* can be confused with *B. semicompositum* (see latter), as both have papillose fruits and other similar features. However, in the latter the ridges of the fruits are not visible and the papillae on the fruit are whitish, while in *B. tenuissimum* ridges are (generally) clearly visible and papillae are brownish.

2) Although *Bupleurum procumbens* Desf. is treated here as a synonym, this taxon has some distinctive features that could merit a subspecific or, at least, varietal rank, as it has been regarded by some authors (e.g. H. Wolff, 1910, & Pottier-Alapetite, 1979). In '*B. procumbens*' the ridges of the fruits are inconspicuous (almost like *B. semicompositum*), but with the remaining features very much the same as the typical *B. tenuissimum*. However, I have only seen a few specimens of this taxon, and to adjudicate an appropriate rank, more material needs to be studied, in particular from Tunisia.

3) Linnaeus validly published the name *Bupleurum tenuifolium* L. in his *Flora Monspeliensis* (ed. 1759). But this new name is likely the result of a 'typographic' mistake, as this taxon corresponds to *B. tenuissimum* L. (Stearn, 1973, p. 631 – see this paper also for full explanation of the link between Linnaeus' *Flora Monspeliensis* and Magnol's *Botanicum Monspeliense*). The description of Magnol's '*Bupleuron angustissimo folio* PIN.' (PIN. = *Pinax* of C. Bauhin, 1623) to which Linnaeus' *B. tenuifolium* refers is undoubtly that of *B. tenuissimum*. The indication of the characteristic late flowering time (September) and the localities cited, Mommau & Gramuntij near Montpellier (ecologically suitable), both support the idea of *B. tenuifolium* being the same as *B. tenuissimum*. Also, the name in C. Bauhin's *Pinax* (1623), referring to '*Bupleurum minimum* Col.', is also an element in the protologue of *B. tenuissimum* L. (see also illustration of '*Bupleurum minimum*' in Colonna, 1606, p. 247).

Typification notes:

1) The original material of *Bupleurum tenuissimum* is as follows: Herb. Burser 16: 11 - practically destroyed - (UPS - IDC microfiche!); Herb. Linn. 116.13 (S - IDC microfiche!); [icon!] '*Bupleurum minimum*' in Colonna (1606, p. 247); [icon!] '*Bupleurum angustifolium folio ...*' in Morison, *Pl. hist. univ.* 3, sect. 9: tab. 12 ['t. 14'], fig. 4 (1699).

2) Below is the typification of *Bupleurum procumbens* Desf. (see taxonomic note 2, above). It is possible that there is an isotype of this taxon in the herbarium of Webb in Florence (FI-W) – see typification notes of *B. plantagineum* Desf., and also Steinberg, 1977, p. 5.

Bupleurum procumbens Desf., *Fl atlant.* 1: 230 (1798).

Type: Holotype – 'Herbier de la Flore Atlantique donné au Muséum par M. Desfontaines N° *Bupleurum procumbens*' (P-Desf.!).

Type locality: "Habitat prope Tunetum".

3) Typification of '*Bupleurum tenuifolium*' (see taxonomic note 3, above) is as follows. Neotype is required as there are no extant elements.

Bupleurum tenuifolium L., *Amoen. Acad.* 4(70): 480 [*Fl. monsp.*] (1759).

Type: Neotype (selected here) – "Flora Galliae et Germaniae exsiccata de C. Billot. 778. *Bupleurum tenuissimum* L. [...] Fleurs le 25 août, fruits le 25 septembre 1851. Bords des levées du Cher, de Roche-Pinard à l'écluse du canal, près de Tours (Indre-et-Loire).", J. Delaunay (BM!).

Type locality: "in vinetis circa la Garrigue de Mommau, & circa pratulum luci Gramuntij Septembri mense cum flore & femine pluries vidimus".

11) *Bupleurum acutifolium* Boiss., *Elench. pl. nov.*: 47 (1838).

Type: Lectotype (selected here) – Herb. Boiss.: "*Bupleurum acutifolium* Boiss. *El.* n° 83", "in dumetis partis inferioris Sierra d'Estepona. Mai 1837." (G-BOIS!). Isolectotypes in E and G-BOIS!

Type locality: [Spain, Málaga] "Hab. in dumetis siccis partis inferioris Sierra d'Estepona. Alt. 1000'-2000'."

Synonyms: *Bupleurum paniculatum* Brot. var. *acutifolium* (Boiss.) Willk. & Lange, *Prodr. fl. hispan.* 3: 74 (1874).

Name origin: From the Latin '*acutus*' (= acute, pointed) and '*folium*' (= leaf), because of its acuminate leaves.

Illustrations: Boiss., *Voy. bot. Espagne* 1(7): tab. 71 (1840).

Perennial herb to *subshrub* 30-100 cm tall, stems herbaceous, woody at the base, little branched, withering after flowering. *Leaves* all similar, (1-)3-16 x 0.1-0.6 cm, herbaceous, sometimes ± coriaceous, amplexicaul, linear to linear-lanceolate, slightly or not attenuate to the base, acuminate, narrow marginal band smooth, parallel-veined, 3-13 veins, clearly visible, slightly raised, without secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal and lateral, the latter smaller; *rays* 3-9(-14), 1-3.2 cm long, subequal, sometimes unequal, slender. *Bracts* 3-5, linear, erect-patent, rarely reflexed, much shorter than the rays, persistent in fruit. *Bracteoles* 3-5, 2-3 x 0.2-1 mm, subequal, linear, acuminate, shorter and narrower or similar in size to flowers and fruits. *Flowers* 5-12 per umbellule; *petals* yellow, sometimes with darker (light brown) mid-vein, inflexed apical lobe entire. *Fruits* clearly pedicellate, pedicels (2-)3-10 mm long; mericarps 3-6 x 0.5-2 mm, oblong to oblong-elliptic, smooth; ridges filiform and smooth.

Chromosome Numbers: 2n = 32 (Cauwet, 1979a).

Ecology: Open mediterranean vegetation, or maquis, rocky places, with quartzite, gneiss and peridotite, and in red-limonite soils overlying serpentine. *Staehelino*

Ulicion baeticae.

Altitude: 150-800(-1200) m. **Flowering time:** (May-)Jun. – Sep.(-Oct.)

World Distribution: Endemic to the Iberian Peninsula.

Distribution in the Iberian Peninsula

Spain: Ma
(Sierra Bermeja).

Portugal: BAL
(Serra do Cercal).

Representative specimens:

Spain: Málaga - Sierra Bermeja, 28.vi.1980, Fernández Casas (MA 310021); border of road [in construction] from Puerto de Peñas Blancas to 'Refugio', 5.ix.1997, S. Neves 64 (COI, E); entre Coín y Mijas, 13.vii.1976, A. Asensi & B. Diez Garretas (MGC 3796); Estepona, a los Reales, 15.vi.1994, A. Aparício *et al.* (MA 543467); Jubrique, 18.viii.1975, Fernández Casas (MA 389395). **Portugal:** Baixo Alentejo - Cercal, 18.vi.1962, Bento Rainha 5477 (COI); Odemira, viii.1917, Ricardo Jorge (E; MA 86575); S. Luis, Serra de S. Domingos, 10.viii.1917, Ricardo Jorge s.n. (COI); Serra de S. Domingos, 'Cargo', 8.viii.1997, S. Neves 27 (COI, E); Serra do Cercal, border of the road from Vila Nova de Milfontes to Cercal, 16.ix.1996, S. Neves 24 (COI, E).

Conservation status:

Bupleurum acutifolium has a very restricted distribution. The Portuguese population is apparently healthy, even considering its very limited area, and does not seem to be in immediate danger. Here, *B. acutifolium* is even found in a densely cultivated area of *Eucalyptus globulus* (a major environmental problem in Portugal). Until my visit in 1996, the species had not been collected nor recorded in the area since the 1960s, and it was a relief to find that it had survived the eucalyptus plantation.

The Spanish population in Sierra Bermeja is potentially endangered. The plant is already rare in the area, and it does not seem to occur in the pine woodlands (*Pinus*

halepensis and *Pinus* spp.) that cover the higher parts of the Sierra. During my fieldwork in 1997 I only found a few plants of *B. acutifolium* growing along margins of the pine woods. A soil densely covered with pine needles, with subsequent acidification of the soil, is probably a major factor inhibiting the growth of this species in these conditions; another might be the shade. A second threat is the potential interest of tourist development in the area. Marbella, and other well-known tourist resorts, are only a few tens of kms from here. Also, the main road going up the 'Sierra' was being resurfaced (probably completely renewed by now), which obviously gives much easier access and causes greater human impact on the area. Unfortunately, Sierra Bermeja does not seem to be under any special legislation for conservation. Even so, the distance from the beach and the fairly steep road may be enough to discourage large scale development of the area.

Critical taxonomic notes:

1) *Bupleurum acutifolium* is closely related to *B. barceloi*, endemic to the Balearic Islands, but the latter is more robust, and its petals have a blackish mid-vein or spot, never seen in material of *B. acutifolium* where petals are always light coloured (pale yellow, sometimes with a light brownish mid-vein). *B. acutifolium* is also morphologically close to some NW African taxa, in particular, *B. oligactis*, but the two species can be more easily distinguished by the margins of the (lower) leaves: smooth in the former, and minutely serrulate in the latter.

2) Material of *B. acutifolium* has been confused with *B. fruticescens* or *B. rigidum* subsp. *paniculatum*. *B. acutifolium* is easily distinguished from *B. fruticescens*, because the latter is a subshrub, with rigid flowering stems, that will become woody, and normally with much shorter leaves, that curve back abruptly at the apex (*B. acutifolium* has ± straight apex). *B. acutifolium* is easily distinguished from *B. rigidum*, because the latter has very hard leaves with very prominent veins.

3) ITS gene sequences of the two populations (Portuguese and Spanish) are more distinct than would be expected for populations of the same species (see chapter 9). Further research is needed to ascertain if the two populations should be regarded as different species, although there is no clear morphological difference.

12) *Bupleurum album* Maire in *Bull. Soc. Sci. Nat. Maroc* 8: 132-133 (1928).

Type: Lectotype (selected here) – Herb. Maire ("Université D'Alger. Herbier de l'Afrique du Nord,"): "M. Grand Atlas: Glaoua, pâturages arides caillouteux dans la plaine de Telouet, au N de la Kasba, 1900 m", 9.vii.1924, R. Maire (MPU!) – Isolectotype at P! (P 84268!) – see typification notes below.

Type locality: [Morocco] "Hab. in pascuis rupestribus nec non glareosis aridis clivi meridionalis Atlantis Majoris, in calvitiis *Junipereti phoeniceae* nec non in *Stipeto tenacissimae*, solo arenaceo nec non calcareo: in ditionis Glaoua glareosis prope castellum Telouet, ad alt. 1750-2000 m (Maire, 1924), prope Tidili [...]; in valle amnis Ziz prope Rich [...]; in valle amnis Gair prope Gourrama, Tijane, et ad radices montium Aït-Mesrouh [...]."

Name origin: From the Latin '*albus*'(= white), because of its 'white' flowers.

Perennial herb to *subshrub* 5-40 cm tall, caespitose (growing in tufts), woody at the base, stems herbaceous, flowering stems withering after fruiting. *Leaves* all similar, 0.3-2 x 0.15-0.3 cm, ± coriaceous, subamplexicaul, linear-lanceolate to oblong-lanceolate, slightly attenuate to the base, acute, shortly mucronate-uncinate, narrow marginal band smooth or sometimes minutely serrulate, parallel-veined, 1-3(-5) veins, visible, without secondary veins, thick intramarginal vein absent; *basal leaves* crowded, withering before or during flowering; *cauline leaves* sparse. *Umbels* terminal and lateral, lateral sessile, all similar, compact; *rays* 1-3(-4), very short (sometimes slightly longer in terminal umbels), 0.1-0.5(-1.9) cm long, subequal, rarely unequal, slender to thick. *Bracts* 1-5, lanceolate, erect-patent, of similar length or longer than the rays, persistent in fruit. *Bracteoles* 4-5, 1.5-2 x 0.4-0.5 mm, subequal, lanceolate to linear-lanceolate, acute to obtuse, similar length and width than flowers or fruits. *Flowers* 3-8 per umbellule; *petals* white or whitish, with or without darker mid-vein, inflexed apical lobe fimbriate. *Fruits* sessile, pedicels 0.1-0.2 mm; mericarps 3-4 x 0.5-1 mm, oblong to oblong-elliptic, smooth; ridges narrowly winged, smooth or slightly crenulate.

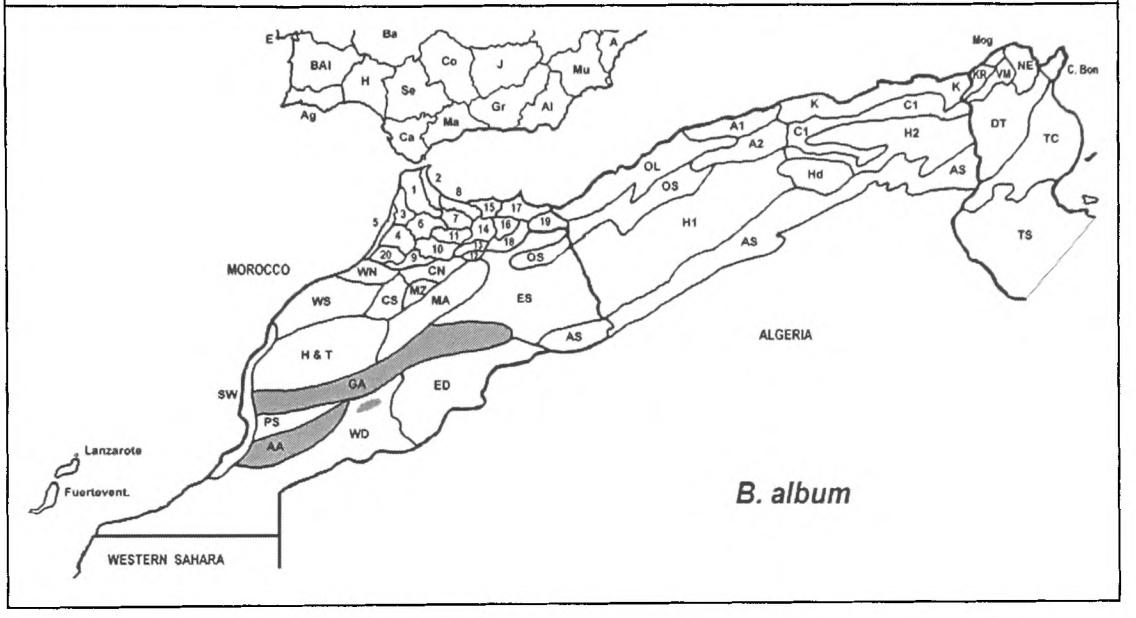
Chromosome Numbers: 2n = 32 (Cauwet, 1979a).

Ecology: Pasture, rocky or stony places, sandy, siliceous or volcanic soils; goat grazed areas.

Altitude: 1300-2000 m. **Flowering time:** May – Jul.(-Aug.)

World Distribution: Endemic to Morocco.

Distribution in NW Africa: *Morocco*: AA, GA, WD (Jbel Sarhro).



Representative specimens:

Morocco: AA - Igherm, rocailles siliceuses, 21.iv.1931, E. Jahandiez (MA 86421); 4 km from Igherm, road to Taliouine, 10.vi.1974, Reading Univ./ BM Exped. 532 (RNG); Mts North of Taznacht [Tazenakht], 8.vi.1936, E.K. Balls B2680 (E, K). **GA** - 40 miles SE of Marrakech, 25.viii.1969, P. Goodchild 103 (BM). **WD** - "Boumalne-du-Dadès [in Jbel Sarhro]: terrain d'aviation", 25.iii.1954, Ch. Sauvage 11925 (MA 325269); Ouarzazate, J. Sarhro, 31 km on track, 41 km from Ouarzazate to Ksar-es-Souk, 18.vi.1974, Reading Univ./ BM Exped. 825 (BM, RNG).

Critical taxonomic notes:

Bupleurum album is very easily recognized by its very compact and axillary umbels that are unique in the genus. Its flowers are also very characteristic: **a)** petals with fimbriate inflexed apical lobe (it has several marginal prolongations); and **b)** its very long styles up to 3 mm (normal length of styles in herbaceous species is 0.05 to 1 mm). I have not been able to confirm that the petals of this species are white, as the colour changes to beige after the specimens are dried. If the petals are indeed white, this will certainly be a good character for identification in the field – the vast

majority of the species in *Bupleurum* have yellow or yellowish-green flowers, a few have purplish or purplish-green flowers, and *B. album* would be the only species with white flowers.

Typification notes:

In the protologue, Maire indicated that the syntypes were in "Herb. Univers. Algeriensis" [= AL] and "Herb. Inst. Imp. Scient. Rabatensis" [= RAB]. So it could be argued that I should select the type from material from one of these herbaria. However, it should be noted that I am selecting the type from the herbarium of Maire (MPU), and that even the label indicates that the specimen had once been in the "Université D'Alger".

The specimens designated here as lectotype (MPU) and isolectotype (P) were once part of the same sheet of the herbarium of Maire in MPU. During my visit to MPU (1996), I noticed that sections of the sheets of several type specimens had been cut out. On questioning this, the curator, P.A. Shäfer, kindly explained that in order to centralize types, a duplicate of every type specimen had been requested by the Paris herbarium (P). However, in some cases there was no duplicate, and the type specimen may have not been sent. But, if the only sheet available had more than one specimen, one was selected, cut out, and sent to Paris.

I found the following syntypes: Glaoua, "pâturages caillouteux [stony], prés de la Kasba de Teluet, 1800-1900 m", 9.vii.1924, R. Maire (BC-Sennen 825950; MA 470497; P 84269 & 84270); "Grand Atlas: vallée de Telouet, 1950 m", 9.vii.1924, R. Maire (MPU); "In pascuis lapidosis prope Aïm-Rich ad radices meridionales Atlantis Majoris", 7.v.1927, R. Maire "Herbier d'la Afrique du Nord" (MPU & P).

13) *Bupleurum balansae* Boiss. & Reut., *Diagn. pl. orient.* Ser. 2, 3(2): 83 (1856).

Type: Lectotype (selected here) – Herb. Boiss.: "482. *Bupleurum fruticescens*, Spr. Coteaux avoisinant la Batterie espagnole.", 30.vi.1952, B. Balansa (G-BOIS!). Isolectotypes at E! and K!

Type locality: [Algeria] "Hab. in collibus propè Arsew Mauritaniae Tingitaniae Reuter, propè Oran loco la Batterie Espagnole dicto cl. Balansa anno 1852 N° 482." (Arzew, Oran Littoral).

Synonyms: *Bupleurum fruticescens* auct. non L., *Cent. pl.* 1: 9 (1755); *B. melillense* Pau in *Bol. Soc. Aragon. Ci. Nat.* 17: 129 (1918); *B. rigescens* Maire & Sennen in Sennen, *Diagn. nouv.*: 192 (1936); *B. fruticescens* L. subsp. *balansae* (Boiss. & Reut.) O.Bolòs & Vigo in *Butl. Inst. Catalana Hist. Nat.* 38, *Secc. Bot.* 1: 83 (1974).

Name origin: Dedicated to the collector, the French botanical explorer Benedict Balansa (1825-1891).

Subshrub 15-70 cm tall, stems becoming woody, ± branched, persistent after flowering. *Leaves* all similar, (0.8-)1.5-8 x 0.1-0.3(-0.5) cm, ± coriaceous, subamplexicaul, linear to linear-lanceolate, sometimes subulate, slightly or not attenuate to the base, acuminate, narrow marginal band generally ± minutely serrulate, parallel-veined, 3-5(-6) veins, clearly visible, slightly raised on both faces, without secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering; *lower leaves* often crowded or tufted; *upper leaves* sparse. *Umbels* terminal and lateral, terminal larger; *rays* 3-7(-10), (0.4-)1-4(-5) cm long, subequal or unequal, slender but ± rigid. *Bracts* 4-5(-7), linear to linear-lanceolate, erect-patent, much shorter than the rays, persistent in fruit. *Bracteoles* 5, 1.5-2(-4) x 0.3-0.8 mm, subequal, linear to linear-lanceolate, acute, shorter and narrower than flowers or fruits. *Flowers* 3-8(-12) per umbellule; *petals* yellow, with darker mid-vein, inflexed apical lobe entire. *Fruits* sessile or subsessile, pedicels 0.3-1 mm long; mericarps 3.5-4(-5) x 0.8-1 mm, oblong-elliptic, smooth; ridges filiform and smooth.

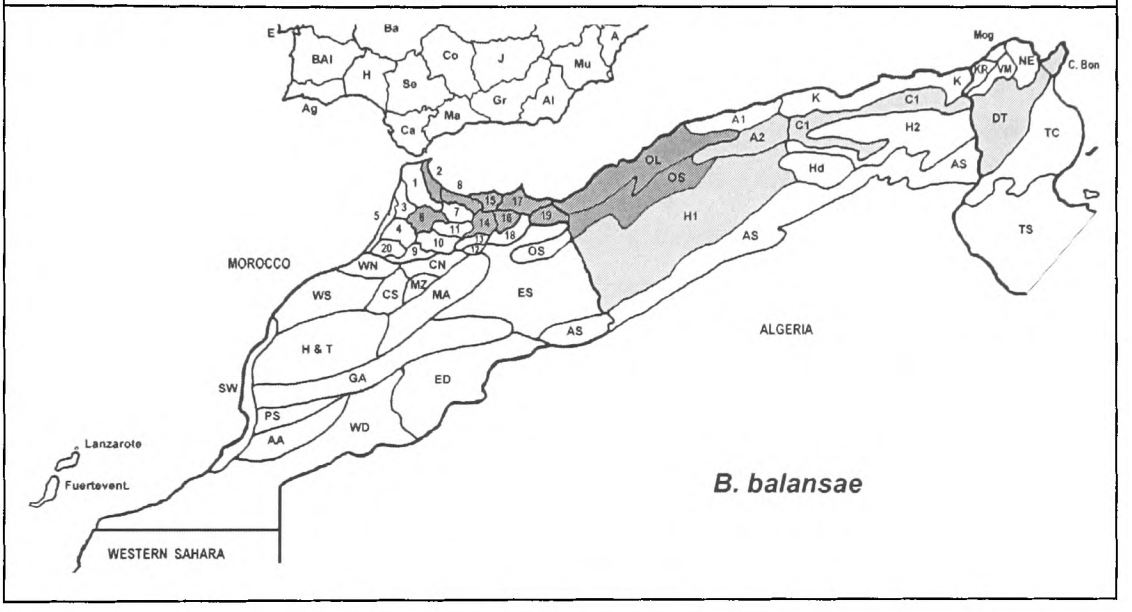
Chromosome Numbers: 2n = 32 (Cauwet, 1979a).

Ecology: Maquis or scrub, rocky places, on limestone or sandstone, slaty, clayey or sandy substratum. *Rosmarino-Ericion.*

Altitude: 190-1600 m. **Flowering time:** Jun. – Aug.

World Distribution: Endemic to NW Africa.

Distribution in NW Africa: *Morocco*: 2, 6, 8, 14, 15, 16, 17. *Algeria*: A2?, C1?, H1(rare), OL, OS. *Tunisia*: C. Bon?, DT.



Notes: According to Quézel & Santa (1963) in their *Nouvelle Flore de l'Algérie*, *Bupleurum balansae* occurs in "all the Tell" [Tell is the name given to the Mediterranean coastal region of 'French' North Africa with a Mediterranean subtropical climate – Seltzer, 1966]. I am not sure of which areas of Algeria these authors considered to be 'the Tell', at least, it seems to include: A2, C1 & OS (= O3 in the *Flora*). However, I have not seen material of the species for these Algerian areas or from Tunisia.

Representative specimens:

Morocco: 2 - Jebel Ghorgez (Beni Hosmar) S of Tetuan, 3.vii.1973, Davis 54719 (E); W Rif, c. 35 km S of Tetouan, near Souk-el-Arba, 18.vi.1987, S. Jury *et al.* 8338 (RNG). **6** - El Zoco Telata Eslef, 12.vii.1930, Sennen & Mauricio (BC 825983). **8** - Targuist, road to Beni-Boufrah, 30.vi.1993, S.L. Jury & L.S. Springate 11324 (RNG). **14** - c. 10 km from Aknoul, Col du Nabor, 17.vi.1992, Optima Iter V 1261 (RNG). **15** - Nador, Djebel Kech Kech, 18.vi.1988, J. Molero *et al.* (BC 814000; RNG). **17** - Muley-Rechid, 20.vi.1931, Sennen & Mauricio (BC 141413, 825985; BM; MA 86590); Montes de Quebdana [Kebdana], ?.vii.1930, Sennen & Mauricio 7579 (BC 825952); Beni-Bu-Yahi, à Tagzuf,

21.vi.1932, Sennen & Mauricio (BC 825954; MA 86580); Melilla, Hidum, 8.vii.1933, Sennen & Mauricio 8801 (BC 825953; BM; MAF 57679). **Algeria: H1** - Hauts plateaux [...?] (Sahara oranais), 21.vi.1968, A. Warion (E, K). **OL** - Oran, plateaux du Santo, 9.vii.1882, O. Debeaux (P); Oran, maquis ou Djebel Santo, 1.x.1884, Debeaux (MA 86577); rochers, près d'Oran, vi.1911, M. Gandoger (BC 25783); Oran, Santa-Cruz, 21.vii.1929, A. Faure (BC 141393; BM; MA 86581); Les Trembles (Departement d'Oran), 4.viii.1929, A. Faure (BC 825969). **OL/OS?** - Oued-Imbert (Oran), 20.vii.1912, A. Faure (MA 86579).

Critical taxonomic notes:

1) The main distinguishing features of *B. balansae*, among other perennial and shrubby N African species, are its sessile or subsessile flowers and fruits, and its leaves with parallel-veins, clearly visible and slightly raised in both faces (cf. with *B. oligactis* and *B. montanum*). However, I am not satisfied with my present definition of this taxon: there is too much polymorphism in shape and length of the leaves, and even how 'clearly raised' are the veins of the leaves (sometimes the same specimen shows variation). Length of pedicels is too often not a reliable character – I would normally avoid it for identification if any other more reliable character was available. Some specimens that I would identify as *B. balansae*, because of its sessile flowers or fruits, seem to be too different from the original material, and, morphologically, are far closer to *B. oligactis*. There is also the possibility that part of this material corresponds to *B. mauritanicum* Batt., a taxon that needs further research to decide its appropriate rank. *B. mauritanicum* seems only to differ from *B. balansae* by the degree of prominence of the veins in its leaves, being less conspicuous in the former.

With the data available, I cannot clearly delimit the variation that occurs in each of the populations that I included in this taxon. The two samples I sequenced of the ITS gene for *B. balansae* (see chapter 9) have more differences than would normally occur in populations of the same species. I am sure that I am including the typical material of *B. balansae*, but I might be including something else too. Detailed field work is necessary to establish the main features in each population of these 'shrubby plants with sessile flowers'; only after this it might become clearer which characters are relevant to distinguish them.

2) *Bupleurum rigescens* Maire & Sennen is here considered a synonym of *B. balansae* Boiss. During my visit to Montpellier herbarium (MPU) in 1996, I found amid herbarium specimens (Herb. A. Faure), what seems to be an extract from a letter written by Maire responding a query about the differences of *B. rigescens*

Maire & Sennen when compared with *B. balansae* Boiss. & Reut. var. *sessile* Clary. Maire's reply was as follow: "[...] Le *B. rigescens* est une initiative de Sennen dans laquelle je ne suis pour rien malgré qu'il ait ajouté mon nom au sien; il y en a d'autres comme cela. Je le mets simplement en synonymie." It seems very clear that Maire strongly disagreed with the 'initiative' of Sennen in publishing this new species including his name in co-authorship. Even Maire considered that the best place for this name was 'in the synonymy' of *B. balansae.*

Typification notes:

1) There are two syntypes of *Bupleurum balansae* Boiss. & Reut. in Geneva (G). The chosen lectotype is a good complete specimen. The other syntype is not a flowering specimen: Herb. Reut. - "Boissier et Reuter. Iter Algeriensi-Hispanicum. *Bupleurum acutifolium* Boiss.! Prov. Oran. Arsew in collibus." iv.1849, G.F. Reuter (G!).

2) The following is the typification of *Bupleurum mauritanicum* Batt. (see taxonomic note 1, above):

Bupleurum mauritanicum Batt. in *Bull. Soc. Bot. France* 10: 386-387 (1888).

Type: Lectotype (selected here) – "M. Battandier Plantes d'Algerie. *Bupleurum mauritanicum* Battand. Mahroun sud Oranais. Dans l'Halfa", vi.1887, Trabut (P!).

Type locality: "In planitiebus excelsis provinciæ oranensis, *Stipa tenacissimæ* L. socia; inter Mahroun et Ras-el-Mâ et ibi ab amicissimo D^re^ Trabut, vigesimo die junii, anno 1887, inventa."

There is a specimen of *B. mauritanicum* Batt. at MPU incorrectly labelled as type: "Université d'Alger. Herbier de l'Afrique du Nord. *Bupleurum mauritanicum* Batt. Type! O. Steppes d'Alfa entre Mahroum et Crampel.", vii [year?], J.A. Battandier (MPU!). The indication in the protologue of *B. mauritanicum* is clear: the type material was collected by Trabut.

14) *Bupleurum barceloi* Coss. ex Willk. in *Linnaea* 40: 83-84 (1876).

Type: Holotype – Herb. Willk.: "Montes de Fornalutx (Barceló)", "7 bre 1870", F. Barceló (COI!).

Type locality: "Mallorca: in fissuris rupium calcar. regionis submontanae in tractu Sierra passim (hucusque non nisi supra pagum Fornalutx pr. Soller et pr. S'Escrop, ubi cl. Barceló d. 7 Sept. 1870 hanc plantam primus legit)."

Synonyms: *Bupleurum fruticescens* L. var. *barceloi* (Coss. ex Willk.) Knoche, *Fl. Baléar.* 2: 232-234 (1922); *B. dianthifolium* Guss. subsp. *barceloi* (Coss. ex Willk.) O.Bolòs & Vigo in *Butl. Inst. Catalana Hist. Nat.* 38, Bot. 1: 83 (1974).

Name origin: Dedicated to the collector, the Spanish botanist Francisco Barceló y Combis (who died in1889).

Illustrations: P. Marès & Vigineix, *Cat. pl. vasc. Baléares*: tab. 4 (1880); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 447 (1990); A. M. Romo, *Flores Silv. Baleares*: 195, tab. 26, fig. 3 (1994).

Perennial herb to *subshrub* (20-)40-100 cm tall, stems herbaceous, woody at the base, little branched, withering after flowering. *Leaves* all similar, (1-)5-18 x 0.1-0.5 cm, herbaceous or sometimes coriaceous, amplexicaul, linear-lanceolate, slightly or not attenuate to the base, acuminate, narrow marginal band smooth, parallel-veined, (3-)5-10 veins, clearly visible, slightly raised, without secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal and lateral, the latter smaller; *rays* (3-)5-12, 0.4-2.6 cm long, subequal, thin but ± rigid. *Bracts* 3-6, linear, erect-patent to reflexed, much shorter than the rays, persistent in fruit. *Bracteoles* 3-5, 1-3 x 0.5-1 mm, subequal, linear, acuminate, shorter and narrower than flowers or fruits. *Flowers* (3-)5-10 per umbellule; *petals* yellow, with a very dark (blackish) mid-vein or spot, inflexed apical lobe entire. *Fruits* clearly pedicellate, pedicels 3-6 mm long; mericarps 4-6 x 0.3-0.5 mm, oblong to oblong-elliptic, smooth; ridges prominent to narrowly winged and smooth.

Chromosome Numbers: 2n = 24 (Cauwet, 1979a).

Ecology: Fissures of rocks on limestone. *Hippocrepidetum balearica.*

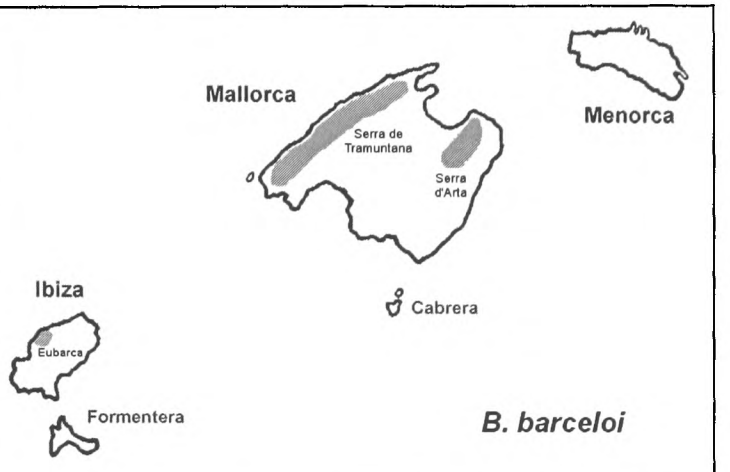
Altitude: 200-1100 m. **Flowering time:** Jun. – Sep.

World Distribution: Endemic to the Balearic Islands.

Distribution in the Balearic Islands:

Mallorca: Mountains of Serra de Tramuntana, from Andratx to Pollença, & Serra de Artà.

Ibiza: Cala d'Eubarca [or Aubarca].



Vernacular names: claveller de penyal, clavellina, clavelliner, clavellinera (Catalán).

Representative specimens:

Spain: *Balearic Islands*. Mallorca - "Fontes des rochers au dessus de Fornalutx", 8.vii.1869, E. Bourgeau (K); rochers de Fornalutx, 18.ix.1870, F. Barceló (G); c. Artà, Atalaia-Moreia 29.vii.1948, Garcías Font (MA 382950); Sa Calobra, 27.iii.1956, O. Bolòs & R. Molinier (BC 135971); Coma d'Eu Arbona, Puig Mayor de Torellas, 22.ix.1932, E.W. Kennedy 55 (K); Bco. de Sóller, v.1899, M. Gandoger (MA 86560); Sóller, rochers, viii.1910, F. Bianor (E; MA 86561); Barranco de Sóller, 3.vii.[19]36, E.W. Kennedy 45 (K); Sóller, 25.vii.1989, J. Orell (G 396478; MA 497522). Eivissa [Ibiza] - Cala d'Aubarca, 5.vii.1920, Font Quer (BC 25761) – only known specimen for this island.

Critical taxonomic notes:

1) *Bupleurum barceloi* is morphologically very close to *B. acutifolium* Boiss., but the latter is less robust, has slender and flexible rays, petals with light coloured mid-vein (brownish, never black), and fruits with filiform ridges.

2) *B. barceloi* is very distinct from *B. dianthifolium* (endemic to the island of Marettimo, S Sicily) and there is no reason to consider these taxa very closely related (no explanation was given either in the publication of the new combination *B. dianthifolium* subsp. *barceloi*). *B. dianthifolium* has much shorter leaves (up to 7

cm long), acute, and fruits with filiform ridges, while *B. barceloi* has very long leaves (up to 18 cm), always acuminate, and fruits with prominent ridges; also the petals of *B. dianthifolium* do not have the characteristic mid blackish vein or spot of *B. barceloi*. Furthermore, chromosome numbers are clearly distinct: *B. dianthifolium*, 2n = 32; *B. barceloi*, 2n = 24 (Cauwet, 1979a).

Typification notes:

There is only one specimen of *B. barceloi* in the herbarium of Willkomm (COI), and its date of collection confirms that this is the specimen mentioned in the protologue. Willkomm interpreted the annotation '7 bre 1870' to mean '7 Sept. 1870', but it is likely that the collector, Barceló, only meant 'Septembre', as in Latin seven is '*septem*' (September being the seven month of the Roman calendar). Although no isotypes have been found, there are several specimens collected by Barceló at the same locality (Fornalutx), and a few of them, in the same month and year as the type are at G & K.

15) *Bupleurum benoistii* Litard. & Maire in *Mém. Soc. Sci. Nat. Maroc* 4(1): 11-12 (1924).

Type: Lectotype (selected here) – Herb. Maire ("Université D'Alger. Herbier de l'Afrique du Nord"): "M. Grand Atlas, Ourika: Tizi-n-Tachdirt pâturages rocailleux porphyriques. 3100-3200 m", 25.vii.1922, R. Maire (P 84265!). Isolectotype at MPU! – basically destroyed; see typification notes below.

Type locality: [Morocco] "Hab. in glareosis porphyricis alpinis Atlantis Majoris, in ditionis Ourika jugo Tachdirt, ad alt. 3150-3200 m, ubi exeunte julio et augusto floret."

Name origin: Dedicated to the French botanist Raymond Benoist (1881-1970), because of his survey of the flora of Morocco.

Perennial herb 2-10(-15) cm tall, caespitose (growing in tufts), with a thick gnarled woody rootstock, stems herbaceous, little branched, withering after flowering. *Leaves* all similar, 1-2(-3.5) x 0.2-0.5 cm, herbaceous, or rarely slightly coriaceous, ± amplexicaul, lanceolate to oblong-lanceolate, gradually attenuate to the base, acute to obtuse, narrow marginal band smooth, more rarely minutely serrulate, parallel-veined, 3-7(-10) veins, visible, without secondary veins, thick intramarginal vein absent; *basal leaves* crowded, persistent during flowering; *cauline leaves* sparse. *Umbels* terminal and lateral, all similar; *rays* 3-5, (0.1-)0.4-1.5(-2.4) cm long, unequal, slender. *Bracts* 3-5, linear-lanceolate, patent to reflexed, shorter to longer than the rays, persistent in fruit. *Bracteoles* 5-6, 1-2 x 0.3-0.5 mm, subequal, linear to lanceolate, acute, shorter and narrower than flowers or fruits. *Flowers* 3-12 per umbellule; *petals* yellow, greenish-yellow, with darker mid-vein, inflexed apex 2-lobed. *Fruits* shortly pedicellate, pedicels 0.5-1.5 mm long; mericarps (4-)5-6(-7) x 1-1.5 mm, ellipsoid, smooth; ridges prominent to narrowly winged and smooth

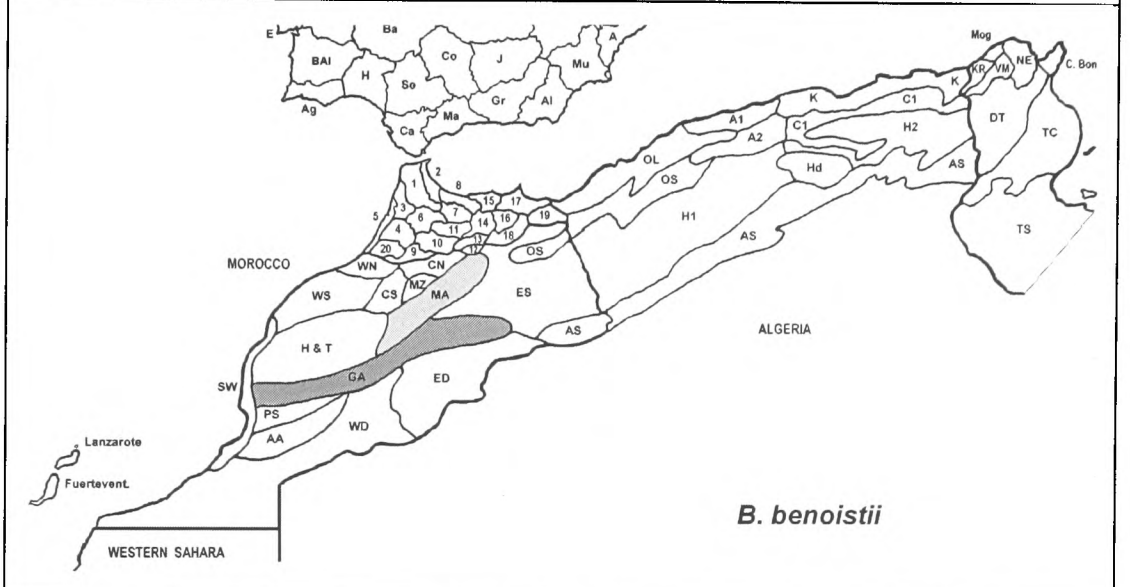
Chromosome Numbers: Not known.

Ecology: Open areas and pastures of high mountains, rocky places, on sandstone and porphyritic rocks, or clayey soils; heavily grazed areas.

Altitude: 2300-3200 m. **Flowering time:** Jul. – Aug.

World Distribution: Endemic to Morocco.

Distribution in NW Africa: *Morocco*: GA, MA.



Note: *B. benoistii* is apparently very rare in the Middle Atlas (MA). I have only seen a specimen collected in this area (see below), and it seems to be from the same and only locality/area cited in the literature: 'Ari Hayan' [*Mém. Soc. Sci. Nat. Maroc* 26: 16 (1930); see also, Jahandiez & Maire (1932, p. 530-531)].

Representative specimens:

Morocco: HA - Oukaïmeden, near Table d'orientation, 20.vii.1973, Davis 55395 (BM, E); Tizi-Oukaïmeden, 20.vii.1973, Davis 55426 (BM, E); above of Oukaïmeden, E of Table de l'Orientation, 18.vii.1981, Davis 67706 (E); Oukaïmeden, 72 km S from Marrakech, 3.vii.1987, S. Jury *et al.* 8858 (MA 391294; RNG); Oukaïmeden, "Aussichtspunkt W des Ortes", 16.vii.1989, D. Podlech 4851 (MA 472361); Oukaïmeden, village and environs, 17.vii.1989, M. Ait Lafkih *et al.* (RNG); S from Marrakech, N end of Jbel Oukaïmeden near azib in ski resort, 29.vii.1997, S. Jury *et al.* 18375 (E). **MA** - "In Atlante Medio prope oppidum Bekrit: in pascuis subalpinis montis Hayan", 2350 m, vii-viii.1924, R. Maire (P).

Critical taxonomic notes:

Bupleurum benoistii, an alpine species with an uncommon habit, can be fairly easily recognised after flowering. The species grows in tufts from a thick and woody rootstock, the appearance is often of a cushion-shaped plant (forming 'domes'), the herbaceous leaves are crowded at the base, and flowering stems are short, rarely

growing more than 10 cm high. Also, the mature fruits are relatively large (up to 6-7 mm long) for such a small plant.

Because of a similar habit, this species might be confused with *B. album* (q.v.), but the flowering stems are completely different. *B. benoistii* might be confused with poorly developed specimens of *B. oligactis* Boiss. (= *B. atlanticum* Murb.), but the leaves of the later are linear-lanceolate and coriaceous, always with a narrow minutely serrulate margin – the margin is normally smooth in *B. benoistii.* Also, flowers and fruits of *B. oligactis* have longer pedicels.

Typification notes:

In the protologue, Maire indicated that the syntypes were in "Herb. Univers. Algeriensis" [= AL], "Herb. Inst. Imp. Scient. Rabatensis" [= RAB] and in the herbarium of Litardière (specimens at G, LAU, MPU, RAB). The specimen was selected from the herbarium of Maire (MPU), and even the label indicates that the specimen had once been in the "Université D'Alger".

The specimens indicated here as lectotype (P) and isotype (MPU) were once part of the same sheet of the herbarium of Maire in MPU – for explanation of this see typification notes of *B. album*. In the case of the type of *B. benoistii*, it was indeed the best specimen that was sent to Paris, as the specimen in MPU is useless for identification: it contains only broken pieces of rootstock and leaves.

16) *Bupleurum canescens* Schousb., *Iagttag. Vextrig. Marokko*: 127 (1800).

Type: Lectotype (designated here) – Herb. Schousb.: "*Bupleurum canescens* Schousb. [illegible] 1872." (C!).

Type locality: [Morocco] "Copiose in fruticeto juxta hortum societatis mercatoriæ prope Mogadore."

Synonym: *Bupleurum handiense* (Bolle) G. Kunkel in *Cuad. Bot. Canaria* 28: 12 (1977).

Name origin: From the Latin '*canescens*' (= becoming grey, greyish), because the surface of the stems looks 'greyish' – in the protologue: '*cortice griseo obductus*' (= cortex with greyish surface). This character is not obvious on dry material, but fresh material is likely to be glaucous and therefore slightly greyish.

Illustrations: H.Wolff in Engl., *Pflanzenr.* 43 (IV.228): 162, fig. 19E (1910); G.Kunkel, *Die Kanarischen Inseln und ihre Pflanzenwelt* (3 ed.): 129 ['abb. 122'] (1993) – as *B. handiense*.

Shrub 100-200 cm tall, stems becoming woody, ± branched, persistent after flowering. *Leaves* all similar, 1-5.8(-8) x 0.3-1.8(-3) cm, ± coriaceous, subamplexicaul, oblong to oblong-lanceolate, sometimes obovate, gradually attenuate to the base, obtuse, mucronate-uncinate, narrow marginal band smooth, parallel-veined, 5-7(-9) veins, visible, sometimes with fine secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal and lateral, similar or terminal larger; *rays* (1-)3-9(-13), (0.3-)1.3-4.2 cm long, subequal, slender to thick. *Bracts* 4-6, ovate-lanceolate to lanceolate, erect to patent, sometimes reflexed, much shorter than the rays, persistent in fruit. *Bracteoles* 5-7, 2-3 x 0.7-1.5 mm, subequal, ovate to ovate-lanceolate, acute, similar length and width than flowers or fruits. *Flowers* 5-15(-20) per umbellule; *petals* yellow, with darker mid-vein, inflexed apical lobe entire. *Fruits* shortly pedicellate, pedicels 1-2.5(-3.5) mm; mericarps 4.5-6 x 1-1.2 mm, oblong-elliptic, smooth; ridges prominent and smooth.

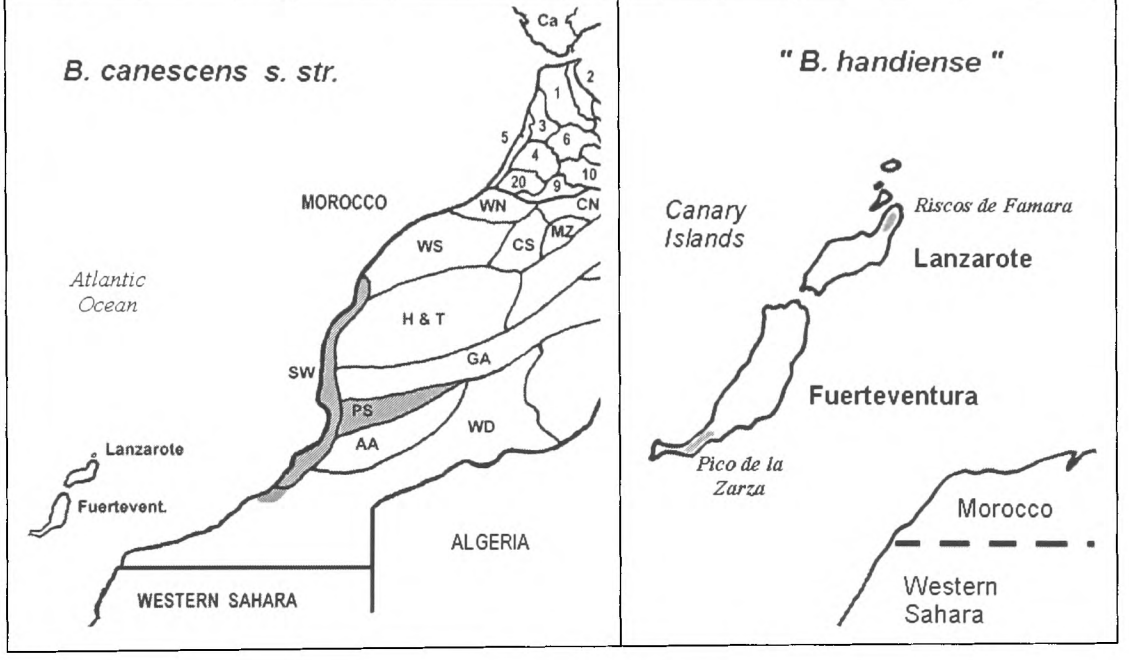
Chromosome Numbers: 2n = 32 (Cauwet, 1979a).

Ecology: Maquis and open vegetation, rocky cliffs and slopes, dry places; on limestone.

Altitude: (0?-)200-600(-860) m. **Flowering time:** (Mar.-)Apr. – Aug.

World Distribution: Endemic to Morocco and Canary Islands.

Distribution in NW Africa:	**Distribution in Macaronesia:**
Morocco: PS, SW, WD (area S of Ifni).	*Canary Isl.*: Fuertev., Lanz.



Representative specimens:

Morocco: PS - Agadir (Sous), rochers calcaires, 2.v.1923, E. Jahandiez 233 (BM, E); Ksima (Sous), terrains secs, 10.v.1923, E. Jahandiez 307 (BC 26031). **SW** - Mogador, v.1871[?], Hooker (LISU 39396G); Mogador [date?], Broussonet (MA 86563); environs d'Agadir, 22.vi.1877, Ibrahim (herb. Cosson) (K); Tarift pr. Idaoua Jssaron, Haha, environs de Mogador, 7.vi.1887, E. Cosson (MPU); Cap Ghir, viii.1930 (cultivated in Alger from seeds collected in viii.1926), R. Maire (BC 825955; BM; MA 86595 & 471291); Ifni, Cerro Bu-Igris, 24.vi.1934, A. Caballero (LISE 17222; MA 86594); Uad Sidi Ifni, 10.iv.1935, Font Quer (BC 812025); Sud-Ouest, *Arganietum maritime*, 10 km Nord d'Agadir, 24.iv.1936, J. Gattefossé (K, MPU); Immouzer Valley, N of Agadir, 28.iii.1972 [not flowering], D. Bramwell *et al.* 265 (RNG). **WD** - Uad Areksis (= Arksis), 12.iv.1935, Font Quer (BC 812027). **Canary Islands:** Fuerteventura - "in praecipicius altis Handiae, El pico de la Zarza", iv.[19]24, O. Burchard 298 (E); Pico de la Zarza, 19.ii.1971 [not flowering], A. Santos (TFC 677); Jandía, 15.vii.1991, LSP, RMC (TFMC 2928). Lanzarote - Roque de Famara, 13.i.1966 [not flowering], K. Lems 6680 (RNG); Peñas de Chache, rare shrub on cliffs above Playa Famara, 15.v.1969, D. Bramwell 1631 (E, RNG).

Critical taxonomic notes:

1) *Bupleurum handiense* (Bolle) G. Kunkel has been regarded in later publications as a distinct species (e.g. Bramwell & Bramwell, 1990; Hansen & Sunding, 1993), but no reasons have been given to support the specific rank. This taxon was first published as: *B. canescens* Schousb. var. *handiense* Bolle in *Bot. Jahrb. Syst.* 14: 241 (1892). The original description was based on material collected in the region of Jandía (or Handia), in the island of Fuerteventura, from where the new name derived. Although Bolle did not make a clear distinction of his new variety, it appears that the main difference of 'var. *handiense*' is that it has much broader [upper] leaves than the typical material of *B. canescens*. In fact, the material I have seen from the population of Fuerteventura has broad oblong to obovate upper leaves, but the material from Lanzarote has leaves that are basically identical to that of *B. canescens*.

Cauwet in her PhD thesis (1976 - 3: 41, 94-96, 127-129) considered that the 'two species', *B. canescens* and *B. handiense*, occurred in the Canary Islands; she indicated as the distinctive feature a larger number of rays, "22-30 rayons", in *B. handiense*, occurring 'only in Fuerteventura', against "3-12 rayons" of *B. canescens*, occurring as she then considered, in Morocco and the Canary Islands (Fuerteventura and Lanzarote). Later, Cauwet & Sunding (1981) considered that all the material from the Canary Islands was of *B. handiense* and that *B. canescens* was an endemic of Morocco; but again no reason was given for this change of opinion.

There is only a small number of herbarium specimens available from '*B. handiense*', which is not surprising considering the limited distribution of this taxon in the wild (see map provided). Nevertheless, I have not found a single specimen with a number of rays near to those indicated by Cauwet (1976) for this taxon – she did not cite herbarium specimens. I cultivated material of '*B. handiense*' (Acc. No 28 - 'seeds from wild sources') in the Jardim Botânico de Coimbra (Portugal), and the plants produced umbels with up to 8 rays, in accordance with the numbers given by authors that know the plants in the wild (cf. Bramwell & Bramwell, 1990, p. 199). The specimen I found with the largest number of rays (13) was actually a specimen of *B. canescens* s. str. [10 km N of Agadir, 24.iv.1936, J. Gateffossé s.n. (MPU)].

Summarising, I have not found any morphological evidence that supports the view of *B. handiense* as a distinct species of *B. canescens*. If broader leaves are a general feature of the population in Fuerteventura, this seems only to merit varietal rank as initially proposed by Bolle, or maybe a subspecific rank if some other 'unique' feature can be identified. The ITS sequences of '*B. handiense*' (Acc. No 28 & 207) and *B. canescens* (Acc. No 301) differ only in a base pair, which is the kind of variation often found between different populations of the same species – see chapter 9 for discussion of molecular data.

2) According to its protologue, *B. oblongifolium* Ball [in *J. Linn. Soc., Bot.* 16: 466-467 (1878)] could be a synonym of *B. canescens*, as the taxon was described as '*fruticosum ramosum, e trunco lignoso*' (referring to its shrubby habit and woody stems) and its oblong and parallel-veined leaves. If this is the case, *B. canescens* might also occur in the High Atlas, at altitudes of up to 1400 m. However, Maire [*Mém. Soc. Sci. Nat. Maroc* 7: 185 (1924)] seemed to believe that this taxon could be a synonym of *B. montanum* Coss., as he noticed that this species also has woody [basal] stems. Indeed, *B. montanum* is woody at the base, and rarely some short basal woody stems are seen in herbarium specimens; but the plant has generally been described as a 'perennial herb' rather than a shrub, as most of its stems are herbaceous. Ball remarked that his *B. oblongifolium* was distinct from *B. montanum* because the latter has '*longius distat ramis* [upper stems] *hieme marcescentibus* [withering], *nec perennantibus*' [not perennial] – so it seems clear he was describing a shrub. Nevertheless, Ball in the same publication also described *B. canescens* as a different species. I have not been able to trace the original material of *B. oblongifolium*: "Aït Mesan circ. 1400 m. Prope Seksaoua [High Atlas], spec. unicum legit J.D.H. [= J.D. Hooker]". Maybe Maire was right and Ball was simply impressed that the plant he saw had a woody branching base, unexpected for the typical herbarium material of *B. montanum*. Retrieving the original material of *B. oblongifolium* or, maybe, field survey of the type locality might clear this doubt.

3) *Bupleurum canescens* can be mistaken for *B. plantagineum* (see taxonomic notes of the latter).

Typification notes:

There is only one specimen of *Bupleurum canescens* in the herbarium of Schousboe (C!) with no indication of date or locality of collection. On the front label is written: "*Bupleurum canescens* Schousb. [illegible] 1872."; and, on the back: "*Bupleurum nervosum* Dn Schousboe e Maroc". However, neither of the handwritings seem to belong to the author (cf. Schousboe's handwriting in Steinberg, 1977, p. 34), and the phrase on the back is unrelated to the protologue. If the number in the front, '1872', is a year, it means that the label was placed many years after the publication of the species, maybe by someone revising or organising the herbarium. This specimen can be regarded as original material because is the only specimen of *B. canescens* in Schousboe's herbarium. However, there is no clear evidence that this was the original specimen used by the author to describe his new species, and therefore is here only designated as lectotype.

17) *Bupleurum dumosum* Coss. & Balansa in *Bull. Soc. Bot. France* 20: 249 (1873).

Type: Holotype – Herb. Cosson: "*Bupleurum dumosum* sp. nov. (Coss.). Partie supériere du Djebel Aït-Ougort prés Keïra, vers 1100 mètr. d'alt." v.1867, B. Balansa (P!).

Type locality: "In montibus ad austro-occidentem urbis Maroc, in parte superiore montis Aït-Ougort prope Keïra, ad 1100 metr."

Name origin: From the Latin '*dumosus*' (= bushy, shrubby), because of its habit.

Subshrub 60-100 cm tall, stems becoming woody, much branched. *Leaves* all similar, growing in clusters, each with (3-)5-15(-20) leaves, upper nodes of flowering stems single-leaved, all leaves 0.5-2.5(-3.5) x (0.05-)0.2-0.4 cm, herbaceous, subamplexicaul, some linear, generally linear-lanceolate or oblong-lanceolate, gradually attenuate to the base (except if linear), obtuse to acute, mucronate-uncinate, narrow marginal band smooth or minutely serrulate, parallel-veined, 3-5 veins, visible, without secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal and lateral, all similar, sometimes lateral smaller; *rays* (2-)3-6, 0.6-2 cm long, generally subequal, slender. *Bracts* (3-)4-5, linear, erect-patent, much shorter than the rays, persistent in fruit. *Bracteoles* (3-)5, 2-3 x 0.3-0.5 mm, subequal, linear, acute to acuminate, shorter or of similar length and narrower than flowers or fruits. *Flowers* (2-)4-6(-8) per umbellule; *petals* yellow, sometimes with darker mid-vein, inflexed apical lobe subentire or slightly 2-lobed. *Fruits* shortly pedicellate or subsessile, pedicels 0.5-1.5 mm long; mericarps 3-4.5 x 0.8-1 mm, elliptic, smooth; ridges filiform and amooth.

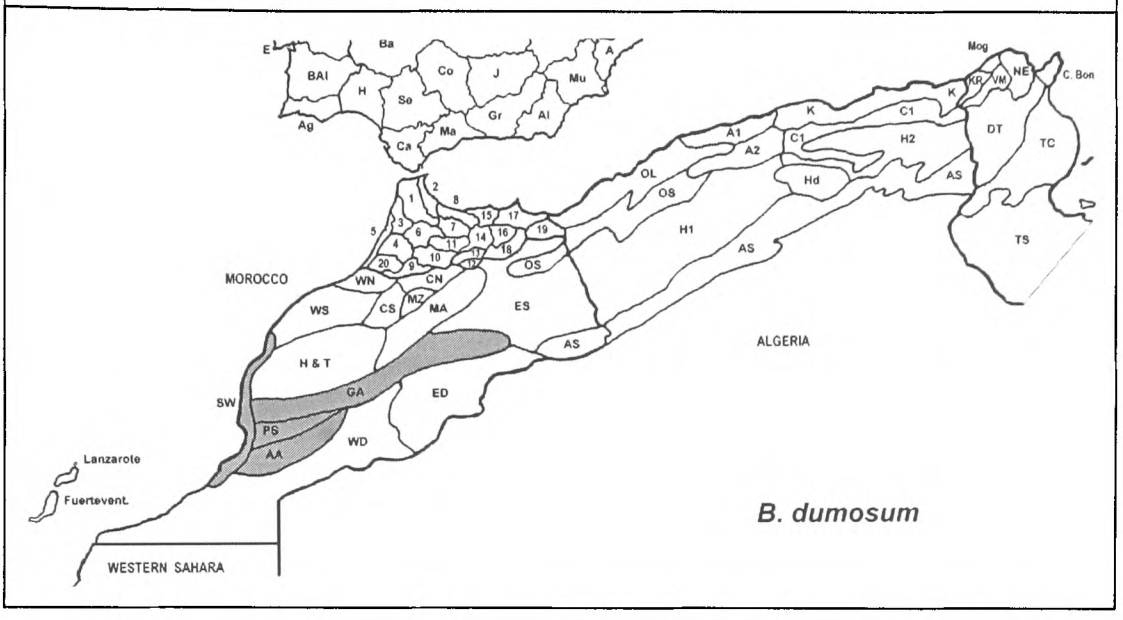
Chromosome Numbers: 2n = 32 (Cauwet, 1979a).

Ecology: Maquis, scrub, woodland or open areas, rocky cliffs or slopes; calcareous, siliceous or slaty soils.

Altitude: (100-)500-1500 m. **Flowering time:** Mar. – Jun.(-Jul.)

World Distribution: Endemic to Morocco.

Distribution in NW Africa: *Morocco*: AA, GA, PS, SW.



Representative specimens:

Morocco: AA - N of Tafraoute, Djebel Lekst, W end, 8.vi.1974, Reading Univ./ BM Exped. 442 (BM, RNG). **GA** - Goundafa, "brousailles prés de Tagadirt-n-Bour", 16.iv.1925, R. Maire (BC 26032; P); "Falaises calcaires de la cascade d'Imouzer des Ida ou Tanane", 5.xii[?].1935, J. Gattefossé (P); 2 km from Asni on road to Tizi-n-Test and Taroudannt, 19.vii.1989, M. Ait Lafkin *et al.* 716 (BM, RNG); c. 9.5 km NNE of Asni, 5 km SSW of Tahanoute, Gorge de Moulay Brahim, 15.iii.1994, S. Jury *et al.* 14157 (RNG). **SW** - Haha, Djebel Amsitten, 24.iii.1931, E. Jahandiez 31 (BM; BC 141403; & MA 86636); Agadir-n-Ighir, 13.iv.1931, R. Maire (BC 141402); N Agadir, Immouzer Valley, 28.iii.1972, D. Bramwell *et al.* 324 (RNG); 5 km above Oulma, valley off road from Tamrhakht to Immouzer, 3.vi.1974, Reading Univ./ BM Exped. 225 (RNG); Agadir, rocailles, 14.iv.1979, C. Vanden Berghen (BM).

Critical taxonomic notes:

Bupleurum dumosum, a shrubby species, is easily recognized by its very characteristic herbaceous leaves in clusters (= bundles). Each cluster has a variable number of leaves, often of different size, some of them much smaller. The upper nodes of the flowering stems are often single-leaved.

Typification notes:

The Balansa specimen indicated as holotype is undoubtedly the specimen cited by Cosson in the protologue. Isotypes are not known.

18) *Bupleurum fruticescens* L., *Cent. pl.* 1: 9 (1755).

Type: Lectotype (selected here) – Herb. Linn. 335.27 (LINN!).
Type locality: "Habitat in in Hispaniae collibus altis."

Name origin: From the Latin '*frutescens*', meaning becoming shrubby.

Subshrub (10-)20-120 cm tall, stems becoming woody, much branched, persistent after flowering. *Leaves* all similar, (0.5-)1-10(-14) x 0.05-1 cm, ± coriaceous, rarely herbaceous, subamplexicaul, linear to oblong-lanceolate, sometimes subulate, slightly attenuate to the base, acute to acuminate, normally uncinate (leaves tips abruptly curving back), narrow marginal band smooth or ± minutely serrulate, parallel-veined, 1-5(-7) veins, visible, sometimes slightly raised beneath, without secondary veins, thick intramarginal vein absent; basal leaves withering before flowering. *Umbels* terminal and lateral, the latter sometimes smaller; *rays* (1-)2-6(-9), 0.5-4 cm long, subequal, slender but rigid to thick and spinescent. *Bracts* (1-)3-5, linear, erect-patent or reflexed, much shorter than the rays, persistent during fruiting. *Bracteoles* (1-)3-5, 0.5-2 x 0.2-0.5 mm, subequal, linear, acuminate, shorter and of similar width than flowers or fruits. *Flowers* (1-)3-6(-8) per umbellule; *petals* yellow or yellow-greenish, sometimes with darker mid-vein, inflexed apex entire to 2-lobed. *Fruits* generally shortly pedicellate, pedicels 1-4(-10) mm long; mericarps 3-6 x 0.8-1.5 mm, oblong to oblong-elliptic, smooth; ridges filiform and smooth.

Chromosome Numbers: 2n = 32 (Cauwet, 1979a).

Altitude: 0-2500(-4000) m. **Flowering time:** (May-)Jul. – Oct. (-Nov.).

World Distribution: C, E & S Spain, and NW Africa.

W Mediterranean Distribution: (see also maps provided for the subspecies).

Spain: A, Ab, Al, B, Bu, Co, CR, Cs, Cu, Ge, Gr, Gu, Hu, J, L, Lo, M, Ma, Mu, Na, Se, Sg, So, T, Te, To, V, Vi, Z. *Morocco*: 2, 7, 14. AA, AS, ES, GA, MA. *Algeria*: A1, AS, H1, H2. *Tunisia*: DT.

Key to identification of subspecies:

1a – Leaves 1-10(-14) cm long, ± coriaceous, sometimes herbaceous. Rays (1-)2-9, slender, semi-rigid, always cylindrical (terete), not spinescent, normally not persistent in consecutive years (easily broken). ..... **a.** subsp. ***fruticescens***.

1b – Leaves 0.5-4(-6) cm long, generally coriaceous. Rays (1-)2-6, thick, stiff, tapering towards the tip, spinescent (looking like spines after the falling of fruits), persistent in consecutive years. ..... **b.** subsp. ***spinosum***.

Critical taxonomic notes:

1) *Bupleurum fruticescens* and *B. spinosum* have been treated as separate species in most taxonomic treatments. These two taxa were first proposed as subspecies of *B. fruticescens* by Bolòs & Vigo (1974), but no explanation was given for this new nomenclatural combination. Nevertheless, my own observations (on herbarium material and during fieldwork) support their view. The Iberian material shows continuous variation between '*B. fruticescens*' and '*B. spinosum*' (intermediate specimens are easily found). The typical cushion-shaped '*B. spinosum*' is normally found in places of high altitude (above 1000 m alt.), often very exposed and under great environmental stress. For example, in Sierra Nevada, *B. spinosum* suffers long periods of frost and snow and, in occasions, strong winds during winter and spring, and high desiccation during the summer. The typical material of *B. fruticescens* is normally found in woods or open areas of lower altitude. During my fieldwork in Spain (1997), I observed that when material of '*B. spinosum*' grows under the shade and protection of a wood, it becomes much more similar to the typical material of *B. fruticescens*, i.e with much longer branches, losing its characteristic cushion-shape, and only the stiff and tapering rays stay ± the same (they grow much longer too). So it seems that the differences between the two taxa are greatly enhanced by environmental conditions. The ITS gene sequence of *B. spinosum* (Iberian material) is identical to that of *B. fruticescens* (see chapter 9), which confirms that the two taxa should be regarded as a single species.

2) *Bupleurum balansae*, a NW African taxon, has been considered very closely related to *B. fruticescens*, and recently was classified as *B. fruticescens* subsp.

balansae (Bolòs & Vigo, 1974). The two taxa are undoubtedly related, but they are sufficiently distinct to be regarded as different species. *B. balansae* has leaves with (generally) ± prominent veins in both faces (veins are only marked or sometimes slightly raised beneath in the leaves of *B. fruticescens*), and with ± straight tips (not abruptly curved back); also the flowers and fruits of *B. balansae* are always sessile or subsessile.

Typification notes:

The original material of *Bupleurum fruticescens* L. is as follows: Herb. Linn. 335.27 (LINN!); [icon] in Barrelier, *Pl. galliam*: tab. 1255 (1714) – information provided by the Linnaean Plant Name Typification Project (Natural History Museum, London).

18a) ***Bupleurum fruticescens*** L. subsp. ***fruticescens***

Illustrations: Cav., *Icon.* 2: tab. 106 (1793); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 446 (1990).

Subshrub up to 120 cm tall. *Leaves* 1-10(-14) cm long, linear-subulate to linear-lanceolate, ± coriaceous or herbaceous. *Umbels* with (1-)2-9 *rays*, slender, cylindrical, ± rigid, not spinescent and not persisting in consecutive years.

Ecology: Mediterranean woods or maqui, scrubs, pastures, in the shade or in the open; generally on calcareous soil, or on gypseous marl or blak clay, dry soils. *Rosmarino-Ericion, Oleo-Ceratonion, Quercetum ilicis.*

Altitude: 0-1600 m. **Flowering time:** (May-)Jul.-Oct.(-Nov.).

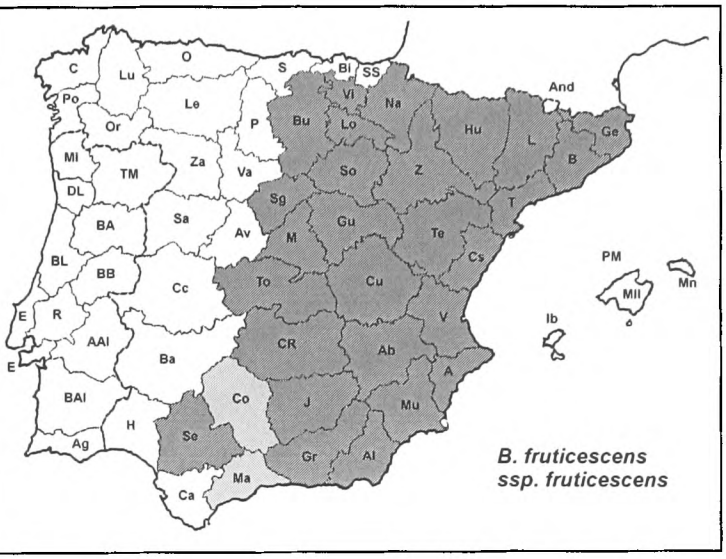
World Distribution: C, E & S Spain.

Note: According to Cauwet (1975b) there are 4 specimens (G-DC and MPU) of *B. fruticescens* labelled as collected between Narbonne and Perpignan (France). Cauwet indicated that she and A. Baudiere searched these area but failed to find

material of this species. Another French locality was cited for *B. fruticescens*, but later the material proved to be of *B. ranunculoides*. Cauwet (1975b) concluded that *B. fruticescens* does not occur in France and that this taxon is 'strictly Iberian'. Fournier (1961) and Tutin (1968) indicate that the French material of *B. fruticescens* is introduced or cultivated.

Distribution in the Iberian Peninsula:

Spain: A, Ab, Al, B, Bu, (Co), CR, Cs, Cu, Ge, Gr, Gu, Hu, J, L, Lo, M, (Ma), Mu, Na, Se, Sg, So, T, Te, To, V, Vi, Z.



Notes: Although cited in the literature, no typical material of *B. fruticescens* subsp. *fruticescens* was seen for the provinces of Córdoba [Rute to river Genil – *Anales Jard. Bot. Madrid* 25: 137 (1967)]; and Málaga [Sierra de Peñarrubia; Sierra de Ojén; Archidona a Loja – *Anales Jard. Bot. Madrid* 25: 119, 125, 137 (1967)].

Vernacular names: botja groga, botja rasparella borda (Catalán); ajocaperdius, cuchilleja, fenoll de rabosa, herba profitosa, hierba cebollá, hierba de la inflamación, hinojo de perro, palito de oro (Spanish).

Representative specimens:

Spain: Alava (Vitoria), Laserna, 28.ix.1983, J. Alejandre & Morante (MA 478800); Alicante, Vall d'Alcala, 29.ix.1995, J. Soler & M. Signes (MA 572400); Albacete, Villa de Ves, 25.v.1991, P. Montserrat (JACA 42691); Almería, Velez Blanco, Sierra de Maria, 26.vii.1981, M. & S. Gardner (SEV 77868); Barcelona, Montserrat, viii.1850, Isern (MA 86627); Burgos, between Zuzones and La Vid, 8.ix.1986, E. Rico (BC 675043); Castellón, Mas del Pont, river Monleón, 20.vii.1986, C. Fabregat (MA 484282); Ciudad Real, Sierra de Alhambra, 14.vi.1935, Gz. Albo (MA 86728); Gerona, Porqueres, 28.viii.1970, O. de Bolòs (BC 605308); Granada, Sierra de Baza, 13.viii.1984, J. Torres *et al.* (GDA 26321); Guadalajara, Hontova, 29.iv.1970, Bellot *et al.* (MA 193557); Huesca, Barbastro, 7.viii.1985, J. Pedrol (MA 313880); Madrid, Dehesa de Arganda, 26.vi.1953, A. Rodriguez (MA 201694); Murcia, Sierra de Espuña, 21.vi.1947, C. Vicioso (MA 86614); Segovia, Sacramenia, 18.vii.1983, T. Romero (GDA 34653); Tarragona, L'Albiol, 10.iv.1993, E. Sobrino (MA 569126); Toledo, Mora, cerro de Buey, 23.ix.1980, P. Cantó & S. Laorga (BCF 42024); Zaragoza, Munébrega, 15.viii.1981, J. Boerboom (MA 535625).

18b) *Bupleurum fruticescens* L. subsp. ***spinosum*** (Gouan) O.Bolòs & Vigo in *Butl. Inst. Catalana Hist. Nat.* 38, *Secc. Bot.* 1: 83 (1974).

Basionym: *Bupleurum spinosum* Gouan, *Ill. observ. bot.*: 8-9 (1773).

Type: Lectotype (selected here) – [icon!]: '*Bupleurum spinosum*' in Gouan, *Ill. observ. bot*: tab. 2, fig. 3 (1773).

Type locality: Not indicated in the protologue.

Name origin: From the Latin '*spinosus*' (= spiny), because its stiff and tapering rays, persistent after fruit dispersal, resemble spines.

Illustrations: Gouan, *Ill. observ. bot.*: tab. 2, fig. 3 (1773); H.Wolff in Engl., *Pflanzenr.* 43 (IV.228): 162, fig. 19A-C (1910); Valdés *et al.* (eds), *Fl. Andalucía Occid.* 2: 312 (1987).

Subshrub pulviniform (cushion-shaped) up to 80 cm tall. *Leaves* 0.5-4(-6) cm long, linear-subulate to oblong-lanceolate, generally coriaceous. *Umbels* with (1-)2-6 *rays*, thick, tapering towards the tip, stiff, persistent after dispersal of fruits, often into the following season, resembling spines.

Ecology: Scrubs, slopes and top of mountains, generally in the open; on limestone, dolomite, slate, eroded substratum. *Rosmarinetalia.*

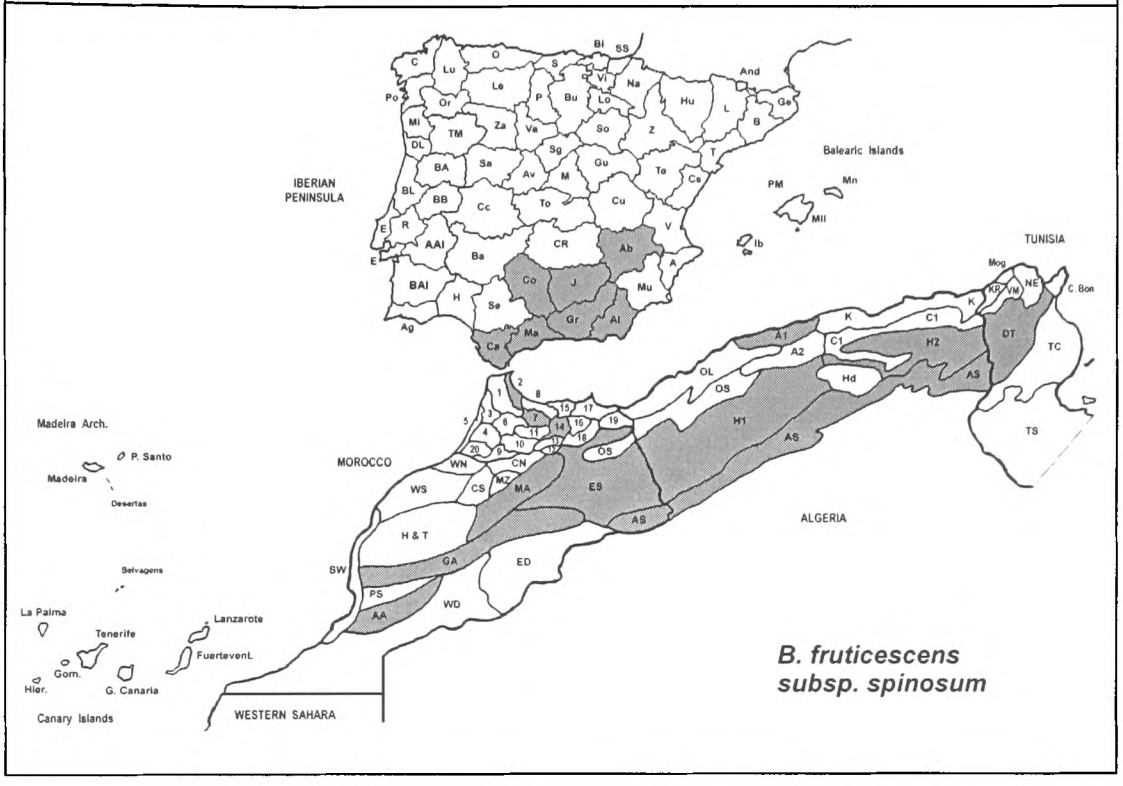
Altitude: 1000-2500(-4000) m. **Flowering time:** Jul. – Oct.

World Distribution: S Spain & NW Africa.

Notes: '*B. spinosum*' has been cited for Corse (= Corsica), although apparently not 'recently' collected (Fiori, 1925). During my visit to the Paris herbarium I examined material of this taxon collected in Corse. Most of these specimens had explicit indication that the material was cultivated. Therefore, it seems likely that all material of '*B. spinosum*' that ever existed in Corse was introduced.

W Mediterranean Distribution:

Spain: Ab, Al, Ca, Co, Gr, J, Ma. *Morocco*: 2, 7, 14. AA, AS, ES, GA, MA. *Algeria*: A1, AS, H1, H2. *Tunisia*: DT.



Vernacular names: choubreq, tafa (Arabic); pelagarco, pendejo (Spanish).

Representative specimens:

Spain: Albacete, Calar del Mundo, 7.ix.1950, S. Rivas Goday & A. Monasterio (MA 310455); Almería, Cerro del Morrón, 10.iv.1994, M. Osorio (MGC 37454); Cádiz, Zahara, Puerto de Acebuches, 21.v.1978, A. Martinez (SEV 84991); Córdoba, Priego, La Tiñosa, 22.ix.1979, J. Muñoz & Tormo (SEV 84761); Granada, Sierra Nevada, Monachil-Dornajo, 14.viii.1988, G. Zúñiga & J. Alejandre (MA 466985); Jaén, Quesada, Puerto de Tiscar, 1.x.1982, C. Soriano & C. Cebollo (MA 462125); Málaga, Junquera, Sierra de las Nieves, 4.vii.1991, B. Cabezudo & J. Nieto (MGC 32803). **Morocco: 7** - AR Rif, 10 km from Ketama, 26.ix.1989, J. Cremades & Buján (MA 530660); **GA** - road to Oukaimeden from Tahanaoute, 3.vii.1987 (BC 688916; MA 391340; & RNG); High Atlas, W face of Djebel Toubkal, Alt. 3500-4000 m, viii-ix.1970, Clayton & Brinklow 64 (E); **MA** - 69 km N of Midelt on P21 road to Meknés, Col du Zad, 13.vii.1987, S. Jury *et al.* 9270 (BC 688380; MA 465283; & RNG). **Algeria: A1** - Chréa, Atlas de Blida, 5.ix.1948, A. Dubuis (MA 489489 & K); Blida to Chrea road, 21.vii.1982, Baxter *et al.* (E); **AS** - El Kantara, 17.ix.1902, L.Chevalier (MA 86557); **AS/ H2?** - Djebel-Tougour, c. Batna, 26.vii.1853, B. Balansa (E).

Typification notes:

I have not found any specimen of '*B. spinosum*' that could be regarded as original material. There is herbarium material from Gouan in Kew (K), but none for this taxon. Therefore, the only original material appears to be the icon referred in the protologue that I have chosen as lectotype.

19) *Bupleurum lateriflorum* Coss. ex H.Wolff in Engl., *Pflanzenr.* 43 (4.228): 160-161 (1910)

Type: Lectotype (selected here) – Herb. Cosson: "*Bupleurum lateriflorum* Coss. Djebel Afougueur, montagne au S.O. de la Ville do Maroc.", viii.1873, Ibrahim (P!). Isolectotype at K!

Type locality: "Marokko: Djebel Ouensa, Djebel Afougueur, im Thale Aït Mesan des Großen Atlas, in einer Höhe von 1200-1600 m".

Name origin: From the Latin '*lateri-*' (= at the side, lateral to) and '*florum*' (of flowers), because of its very characteristic lateral umbels that look almost axillary on the sides of the flowering stems.

Subshrub 50-80 cm tall, stems becoming woody, little branched, persistent after flowering. *Leaves* all similar, (0.5-)1-5.8 x (0.1-)0.5-1.3(-1.7) cm, herbaceous or slightly coriaceous, subamplexicaul, obovate to oblong-lanceolate, gradually attenuate to the base, obtuse to acute, shortly mucronate (slightly uncinate), narrow marginal band minutely serrulate, parallel-veined, 5-7(-9) veins, without secondary veins, clearly visible, thick intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal and lateral, the latter smaller, shortly pedunculate; *rays* 3-11, 0.3-1.5(-1.8) cm long, subequal, slender. *Bracts* 5-6, linear-lanceolate, erect to patent, shorter than the rays, persistent in fruit. *Bracteoles* (4-)5, 1-2 x 0.3-0.5 mm, subequal, ovate-lanceolate to lanceolate, acute, shorter and narrower or of similar width than flowers or fruits. *Flowers* 3-12 per umbellule; *petals* yellow, without darker mid-vein, inflexed apical lobe entire or slightly 2-lobed. *Fruits* shortly pedicellate, pedicels 1-2.5 mm long; mericarps 4-5 x 1-1.5 mm, oblong, smooth; ridges narrowly winged and smooth.

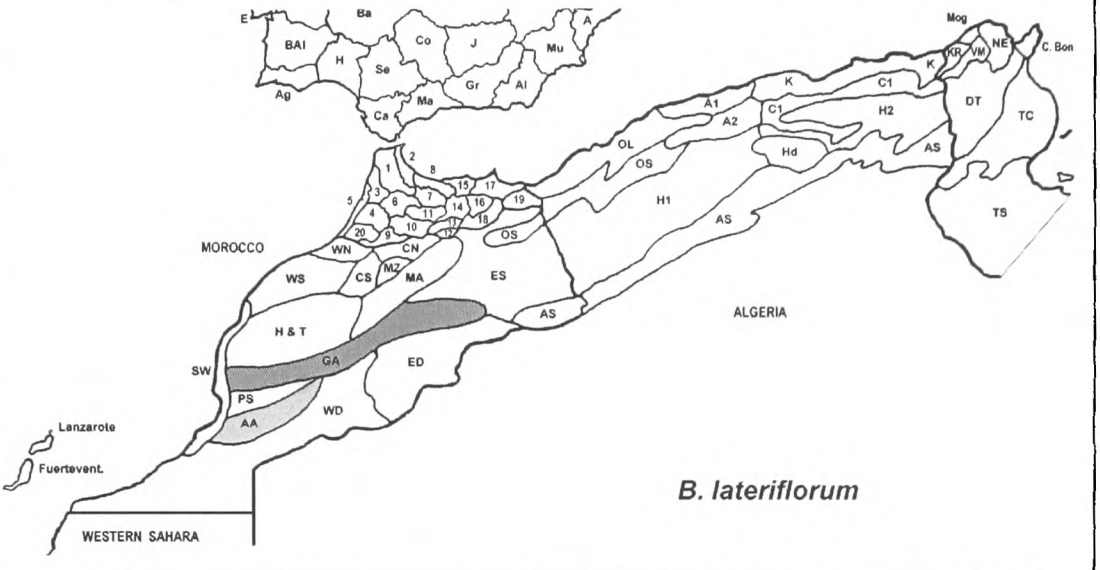
Chromosome Numbers: 2n = 28 (Cauwet, 1979a).

Ecology: Maquis, scrubs or woods, cliffs and rocky places; on sandstone, igneous rock, calcareous or siliceous soil.

Altitude: 1200-2400 m. **Flowering time:** Jul. – Oct.

World Distribution: Endemic to Morocco.

Distribution in NW Africa: *Morocco*: AA?, GA.



Note: All the herbarium material revised had been collected in the High Atlas (GA). However, the species was also cited for the Anti-Atlas mountains (AA) by Jahandiez & Maire (1932); this information needs confirmation.

Representative specimens:

Morocco: GA - "Summitis de l'Atlas au Sud de la ville de Maroc", 15-25.viii.1873, Ibrahim (K, P). Gorges Imi n'Ouaka (Dj. Ghât), 3.viii.1935, J. Gattefossé (BC 86401; MAF 52931 & 59004); around K Asni, 5000 ft, 19.vii.1936, E.K. Balls B3139 (E); Imlil (Asni), 19.ix.1982, J. Lewalle 10549 (BM); 7 km from Asni on road to Imlil, 19.vii.1989, M. Ait Lafkih 696 (MA 499553; RNG); 59 km from S from Marrakech, 14 km due of Asni, 3.vii.1987, S.L. Jury *et al.* 8831 (BM; BC 688917; MA 391338; RNG); Tizi-n-Test, 16.viii.1951, O. Polunin 2051 (BM); Tizi-n-Test Pass, 2.x.1991, M. Ait Lafkih *et al.* 4939 (E, RNG); J. Toubkal, at Sidi Chamarouch, 24.vii.1973, Davis 55520 (BM, E); S from Marrakech, 4 km below Oukaïmeden on road to Vallée de l'Ourika, 28.vii.1997, S.L. Jury *et al.* 18323 (E).

Critical taxonomic notes:

1) *Bupleurum lateriflorum*, a shrubby species, is easily recognizable by its characteristic flowering stems: a larger terminal umbel, with its much smaller, almost axillary lateral umbels (one per upper node) – peduncles of lateral umbels are very short.

2) If not in flower the species might be mistaken for *B. canescens*, another shrubby species with similarly shaped leaves. But the two species can be

distinguished by the narrow marginal band of the leaves: it is minutely serrulate in *B. lateriflorum*, whilst smooth in *B. canescens*.

Typification notes:

Cosson published the name of this species without description in 1875 (*Bull. Soc. Bot. France* 22: 59). He cited a specimen from Djebel Afougueur (High Atlas), collected by Ibrahim. H. Wolff (1910) validly published *B. lateriflorum*, adding another location to the type locality (Djebel Ouensa, High Atlas), but he did not directly cite any specimen. However, Wolff mentioned Cosson's publication, and so, indirectly, the specimen of Ibrahim (Herb. Coss. - P!) can be regarded as original material, and therefore is chosen here as the lectotype.

20) *Bupleurum montanum* Coss. in *Bull. Soc. Bot. France* 3: 706-707 (1856).

Type: Lectotype (selected here) – "E. Bourgeau Pl. D'Algérie 1856. Forêt de cèdres de Teniet el Haad, prov. d'Alger.", 23.vii.1854, E. Cosson (P!). Isolectotypes in in E!, K! & P!

Type locality: "In sylvaticus vel dumetosis regionis montanæ mediæ: in provincia Cirtensi in monte Djebel Tougour! prope Batna; in provincia Algeriensi in sylva cedrorum supra Teniet el Haad!"

Synonyms: *Bupleurum baboranum* Debeaux & E.Rev. ex. H.Wolff in Engl., *Pflanzenr.* 43 (IV.228): 163 (1910) – pro syn., nom. inval. [see *Code* Art. 34.1(c)]; *B. faurelii* Maire in *Bull. Soc. Hist. Nat. Afr. Nord* 30: 344 (1944).

Name origin: From the Latin '*montanus*', meaning it grows in mountains.

20) *Bupleurum montanum* (Cont.)

Perennial herb to *subshrub* 50-150 cm tall, woody at the base, stems herbaceous, little branched, withering after flowering. *Leaves* all similar, 1.3-15(-18.5) x (0.1-)0.3-1.3 cm, herbaceous, rarely slightly coriaceous, subamplexicaul, linear-lanceolate to lanceolate, slightly attenuate to the base, obtuse to acute (rarely acuminate), sometimes mucronate, narrow marginal band smooth, more rarely ± minutely serrulate, parallel-veined, 3-9 veins, visible, sometimes slightly raised beneath, sometimes with inconspicuous secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering; *lower leaves* with shorter internodes, rarely crowded; *upper leaves* sparse. *Umbels* terminal and lateral, terminal larger; *rays* 3-9(-15), 0.5-2(-4.2) cm long, unequal, sometimes subequal, slender. *Bracts* 4-5(-8), linear to linear-lanceolate, erect-patent, much shorter than the longest ray, persistent in fruit. *Bracteoles* 5-6(-8), 1.5-4 x 0.3-0.5 mm, subequal, linear, acuminate, of similar length and width or shorter and narrower than flowers or fruits. *Flowers* (1-)3-11(-20) per umbellule; *petals* yellow or greenish-yellow, with darker mid-vein, inflexed apical lobe entire or subentire. *Fruits* generally short pedicellate, pedicels 1-4.5(-7) mm long; mericarps (4-)5-6.5(-7) x 1-1.5 mm, oblong to oblong-elliptic, smooth; ridges narrowly winged and smooth.

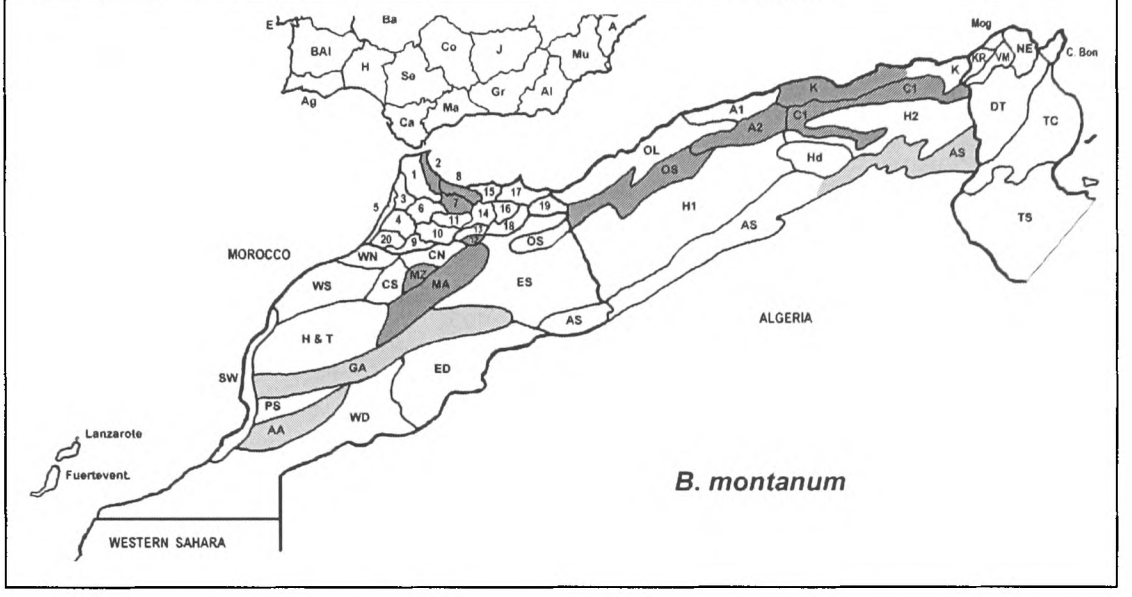
Chromosome Numbers: 2n = 28, 30, 32 (Cauwet, 1979a).

Ecology: Woodland or maquis, sometimes pasture; rocky places, on limestone or schist. Forests with *Abies pinsapo*, *Cedrus atlantica*, *Quercus alpestris* and *Quercus ilex*.

Altitude: 1000-2300 m. **Flowering time:** Jun. – Sep.

World Distribution: Endemic to Morocco and Algeria.

Distribution in NW Africa: *Morocco*: 2, 7 , 8, 12. AA, GA (rare), MA, MZ. *Algeria*: A2, AS, C1, K, OS.



Notes: Although cited in the literature, I have not seen material of *B. montanum* from the Anti-Atlas mountains (AA: 'Mont Kest'– Emberger & Maire, 1941, p. 1084) or from the Saharan Atlas (AS) in Algeria (Quézel & Santa, 1963, p.655).

Representative specimens:

Morocco: 2 - Tetouan, c. 40 km from Chefchaouen, 10 km above Talenbote, on route to Jbel Tassaot, 25.vi.1992, Optima Iter V 2102 (RNG); Chefchaouene, Djebel Bouhalla, 25.vii.1995, M.A. Mateos al. 7219/95 (SEV, RNG). **7** - Chefchaouen, between Ketama and Bab-Berret, 3.xi.1993, P. García Murillo *et al.* ST 252/93 (SEV). **8** - Bu-Meziat, 30.vi.1927 (RNG; SEV 808630). **12** - Approx. 26 km from Taza, near Bab-Bou-Idir, 9.vii.1989, S.L. Jury *et al.* 132 (RNG). **GA** - Djebel Bonachfal, in provincia Demnat, 3.viii.1882, Ibrahim (P); Tizi-n-Test, 16.viii.1951, O. Polunin 2050 (BM); Vallée de Toutline, Sud-Est de Demnat, 1.viii.1935, J. Gattefossé (MAF 58998). **MA** - Daïet Achlef, 13.vi.1923, E. Jahandiez 523 (BC 26036; LISU G39385); Timhadit, au Djebel Tisdadine, 11.vii.1924, E. Jahandiez (E); 4 km above Azrou on road to Midelt, 12.vii.1989, S.L. Jury *et al.* 316 (BM, RNG). **MA/Z** - Azrou to Ain Leuh, 12.vii.1973, Davis 55156 (BM, E). **Algeria: K** - Mont Babors, les rochers escarpés et calcaires, vii.1898, E. Reverchon 319 (BC 825951; BM; E; MA 86570; RNG); Bouira, Parc national du Djurdjura, flanc S du Ras Tigounatine, 25.vii.1985, H. Maurel & R. Rahmoun (BC 688224; MA 366987; MAF 130363), *idem*, 12.ix.1985, H. Maurel & R. Rahmoun (BC 807302; MA 465282; MAF 141124). **OS** - Oran prov., Terny, broussailles vers le Nador, 10.viii.1932, A. Faure (BC 79210; E; MAF 52939). OS? - Oran, Le Ghar-Rouban, broussailles, 2.vii.1933, A. Faure (MA 86569).

Critical taxonomic notes:

1) *Bupleurum montanum* is a fairly polymorphic species, and it is not always easily distinguishable from closely related species, in particular *B. oligactis* Boiss. (= *B. atlanticum* Murbeck). Both species are perennial, with woody base (sometimes

with short, woody basal branches), and herbaceous flowering stems. In general, *B. montanum* has soft, herbaceous leaves, often obtuse (sometimes with a mucro), with smooth marginal band, and flowers and fruits have short pedicels (normally, 1-4.5 mm long). In contrast, *B. oligactis* has coriaceous leaves, generally acuminate, with minutely serrulate marginal band, and flowers and fruits have longer pedicels (normally 4-8.5 mm). However, none of these characters is totally reliable. A few specimens of *B. montanum* have leaves that can easily be regarded as coriaceous, and if they are narrower than usual, they are likely to be acute or acuminate, and the margin is in some occasions minutely serrulate; also a few specimens have longer pedicels than the norm. The texture of the leaves might be, in some degree, affected by environmental conditions; it is possible that the slightly coriaceous leaves are found in material growing in exposed habitats instead of from the protection of a wood. Typical material of *B. montanum* seems to be more often found in woods — unfortunately, many specimens are poorly labelled, so this needs confirmation. I have only seen a few good fruiting specimens for both species, and their fruits seem fairly different: they are slightly larger and have narrowly-winged ridges in *B. montanum*, while ridges are filiform in the fruits of *B. oligactis*. Nevertheless, it is necessary to study more fruiting material, especially from *B. oligactis* to confirm this small difference. The ITS sequences from *B. montanum* and *B. oligactis* are distinct only in c. 6 base pairs (see chapter 9), which confirms that the two taxa are very closely related.

2) *B. montanum* may also be confused with some material of *B. balansae*, but the latter species always has sessile or subsessile flowers and fruits, and the leaves are ± coriaceous, acuminate, with veins slightly raised on both surfaces, and lower leaves are often crowded. *B. balansae* is also a more typical shrub as it has longer woody persistent stems.

3) *Bupleurum faurelii* Maire does not have any distinctive morphological feature and the original material can be easily included within the variation of *B. montanum* Coss. Maire noticed that the vallecular vittae (secretory canals in the fruit) of this taxon were small, and that commissural vittae were absent. However, there is no fruiting specimen in the original material (MPU), and therefore this small anatomical difference cannot be verified.

4) *Bupleurum oblongifolium* Ball. [in *J. Linn. Soc., Bot.* 16: 466-467 (1878)]

could be a later synonym of *B. montanum* Coss., but it could also be a synonym of *B. canescens* Schousb. – see taxonomic notes of the latter species.

5) Three different chromosome numbers have been recorded for *B. montanum* (Cauwet, 1979a). Further research is necessary to confirm these numbers and to ascertain how this variation affects the phenotype; it could be one of the causes of the polymorphism of the species.

Typification notes:

1) The specimen selected here as the lectotype of *Bupleurum montanum* is undoubtedly from the collection used by Cosson to describe his new species: it was collected by the author in the type locality, and on the date indicated in the protologue: "23ª die julii 1854 floriferum lectum." It is also a typical specimen with soft leaves as remarked by Cosson: "diffère par la consistance molle [soft] des feuilles [leaves]". This specimen belongs to the herbarium of Cosson that has been incorporated in the general herbarium in Paris (P). There are several isolectotypes in P!, there is also a isolectotype in E!, and another in K!

2) The following is the typification of *Bupleurum faurelli* Maire, here regarded as a synonym of *B. montanum* Coss. (see taxonomic note 3 above):

Bupleurum faurelii Maire in *Bull. Soc. Hist. Nat. Afr. Nord* 30: 344 (1944).

Type: Lectotype (selected here) – Herb. Maire: "Pentes schisteuses près du part[?] Saint Honoré vers 2000 metres. Grand Atlas Oriental, region d'Agardin[?]", 23.vii.1938, L. Faurel (MPU!).

Type locality: "Grand Atlas oriental: rochers calcaires de l'Akka-n-Ouyad, vers 2000 m (L. Faurel)."

There is another specimen in Maire's herbarium (MPU) that was collected in the type locality: "In cedretis faucium Akka-n-Ouyad Atlantis Majoris, 2000 m, solo calcareo rupestri", 28.vi.1939, R. Maire & M. Weiller 708 (MPU!). This specimen could be regarded as the type, as it was considered by Panelatti (1959, p. 54); however it was collected by Maire & Weiller (in the same year as the publication of *B. faurelii*) and Maire clearly cited a specimen collected by Faurel – although not explicitly mentioned, the author obviously dedicated his new species to the collector.

21) *Bupleurum oligactis* Boiss., *Diagn. pl. orient.* Ser. 2, 3(2): 84 (1856).

Type: Lectotype (designated here) – Herb. Delessert: "881. *Bupleurum paniculatum*, Brot. (Coss.). Pente est du Djebel-Tougour, près Batna", 9.vii.1853, B. Balansa (G-DEL!) – incomplete specimen, see isolectotypes at E! and K!

Type locality: "Hab. in declivitate orientali montis Gebel Tongaur propè Batna Mauritaniæ orientalis cl. Balansa pl. exs. anni 1853 N° 881."

Synonym: *Bupleurum atlanticum* Murb. in *Lunds universitets årsskrift* Afd. 2, Sectio 2: 47-48 (1905).

Name origin: From the Greek 'oligos' (= little, small, few) and '*actis*' (= ray), because its umbels have a small number of rays (1-5, rarely 6).

Illustrations: Murb. in *Lunds universitets årsskrift* Afd. 2, Sectio 2: tab. 9 (1905) [as *B. atlanticum*].

Perennial herb to *subshrub* 30-80 cm tall, stems herbaceous, woody at base, ± branched, withering after flowering. *Leaves* all similar, (1.5-)3-10.5 x 0.12-0.45 cm, coriaceous, subamplexicaul, linear to linear-lanceolate, slightly or not attenuate to the base, acuminate, narrow marginal band minutely serrulate (at least in the lower leaves), parallel-veined, 3-8 veins, visible, sometimes slightly raised on both faces, without secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering; *lower leaves* sometimes crowded; *upper leaves* sparse, sessile. *Umbels* terminal and lateral, all similar or terminal larger; *rays* 1-5(-6), 0.5-4 cm long, subequal or unequal, slender. *Bracts* 1-4, linear, erect-patent, much shorter than the rays, persistent in fruit. *Bracteoles* 4-5, 1-2 x 0.15-0.2 mm, subequal, linear, acuminate, shorter and narrower than flowers or fruits. *Flowers* 4-8 per umbellule; petals yellow, generally with darker mid-vein, inflexed apex entire or 2-lobed. *Fruits* often long pedicellate, pedicels 2-8.5 mm long; mericarps 4-5 x 0.9-1.1 mm, oblong, smooth; ridges filiform and smooth.

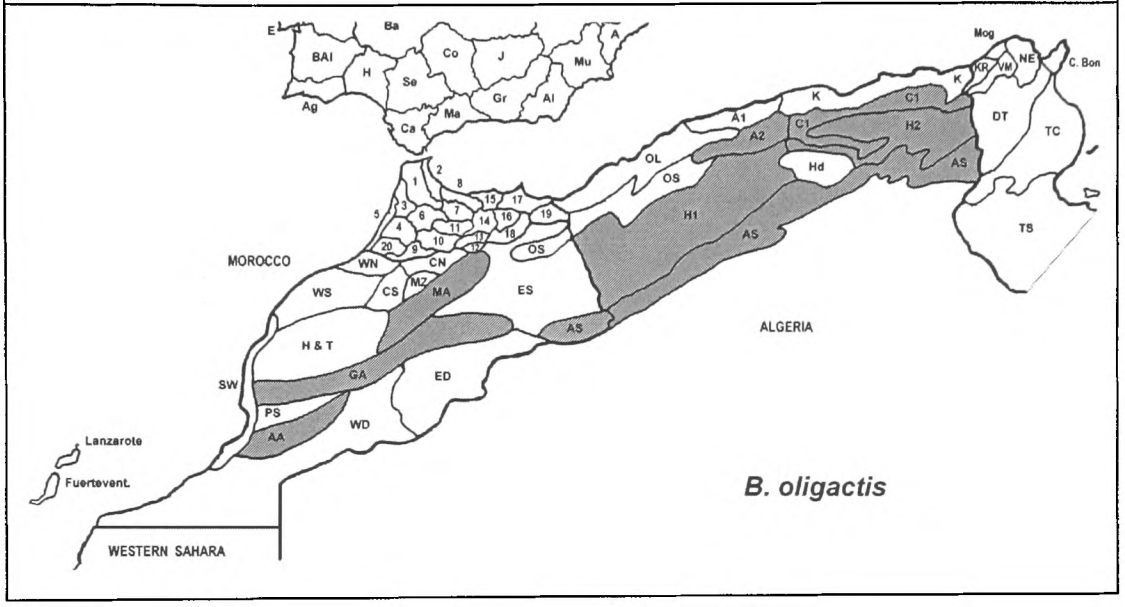
Chromosome Numbers: 2n = 28, 30, 31, 32 (Cauwet, 1979a – also as *Bupleurum atlanticum*).

Ecology: Open vegetation of high altitude, scrub or grassland, more rarely in woods, rocky places; on calcareous or siliceous soil.

Altitude: (?-)2000-3000 m. **Flowering time:** (May-)Jun. – Aug(-Sep.).

World Distribution: Endemic to Morocco and Algeria.

Distribution in NW Africa: *Morocco*: AA, GA, MA. *Algeria*: A2, AS, C1, H1, H2.



Notes: Distribution of *B. oligactis* in Algeria needs confirmation as only a small number of specimens were studied and there has been considerable confusion in the application of this name.

Representative specimens:

Morocco: AA - 28 km Tafroute, road to Igherm, 5 km past road to Ait Baha, 9.vi.1974, Reading Univ./ BM Exped. 484 (RNG). **GA** - Tansrart (Reraïa), pentes pierreuses au Djebel Tihalatine, 7.vi.1921, E. Jahandiez 638 (BC 26029); Vallée de Toutline, Sud-Est de Demnat, 1.viii.1935, J. Gattefossé (BC 86400). Bou Gamez [?], Taorirt n'Ait Milch, 14.viii.1951, D.H.N. Spence S260 (E); Assif-n-Arous, 3 km S of Ait Said, 16.viii.1975, P. Crane (R.U. Biol. Exped) 3 (RNG); 111 km from Errachidia (Ksar-es-Souk) along P21 road to Midelt, 12.vii.1987, S.L. Jury *et al.* 9240 (BC 688369; BM; MA 391152; RNG; SEV 127166); c. 53 km NE from Midelt, below Itzer, 25.ix.1991, M.F. Gardner *et al.* 4846 (E; MA 511293). **MA** - Timhadit, vallée du Guenfoud, 12.vii.1924, E. Jahandiez 809 (BC 26030; BM); 50 miles SW of Midelt, 16.viii.1968, P. Goodchild 29 (BM); d. Beni-Mellal, Sidi-Mohamad To Zaouia Ahanesal, 13.vii.1973, Davis 55183 (BM, E); road to Ifkerne from Boulimane, 17.vii.1976, C.J. & A.R. Humphries 53 (BM); Immouzer des Marmouche, 16.vi.1980, J. Lewalle 9560 (BM). **Algeria: A2** - Djebel Tagga, sur le chemin de Boghar à Teniet-el-Haad, 12.vii.1857, O. Debeaux (E - specimen on the right; P); circa Boghar (Alger) in sylva pini alepensis, vii.1857, O. Debeaux (BC 654723). **AS** - Djebel Ksel prés Géryville Sud de la prov. d'Oran, 30.v.1856, E. Cosson (P). **C1** - 10 km of Batna, Kasrou, 25.iv.1976 [not flowering], D.A. & S.J. Sutton 641 (RNG).

Critical taxonomic notes:

1) For a considerable time I was not sure to which plants the name *Bupleurum oligactis* Boiss. should be applied. It was a disappointment to see that the type specimen from G was useless for identification. However, I later found two isolectotypes (E & K) in good condition, and that, in my opinion, correspond to the same species as the material generally regarded as *B. atlanticum* Murb. That being the case, *B. oligactis* Boiss. as an earlier name has priority. In the protologue, Murbeck pointed out that *B. atlanticum* was different from *B. oligactis* Boiss. because his new species had longer pedicels and the ridges of the stems were prominent and "verrucose", while they were smooth in the latter. However, pedicel length is quite variable and there is no clear gap in the values. The stems of the type material of *B. oligactis* are, indeed, quite smooth, but prominence and roughness of stems seems to be very plastic characters, with differences being found even between stems of a single specimen. Considerable variation in these characters is also found in the type material that Murbeck cited for *B. atlanticum* (see below). ITS sequences obtained from samples of specimens with rough (Acc. 281) and smooth (Acc. 298) stems are identical (see chapter 9).

2) *Bupleurum oligactis* is not always easily distinguished from *B. montanum* (see taxonomic notes of the latter).

Typification notes:

1) The type specimen of *B. oligactis* in Geneva (G-DEL) is basically useless for identification as it only contains stems with no leaves, and only part of an umbel in the capsule. It is chosen here as the lectotype as it was the material that Boissier would have used to describe his new species. However, the isolectotypes in E and K are good complete specimens that support the application of the name.

2) Below is the typification of *B. atlanticum* Murb. here regarded as a synonym of *B. oligactis* Boiss (see taxonomic notes above). Cauwet (1977, p. 169) proposed as lectotype of *B. atlanticum* a specimen in Murbeck herbarium (LD) and cited a isotype in Paris (P). The curator of the Lund herbarium, Per Lassen, has kindly informed me that such a specimen is not at LD. He also remarked that there is no

evidence that the specimen had ever been there, or any of the other specimens cited by Murbeck in the protologue of *B. atlanticum* (6 syntypes). Cauwet (1975, p. 5-6) seemed to believe that all the syntypes were in LD, but she did not mention the 'LD specimen' in the material she revised (Cauwet, 1977, p. 171) It is clear that she designated a specimen she did not see! Per Lassen also added that it is likely that Murbeck would have studied the type material in the Paris herbarium (P). Indeed, I found in P material of 4 of the syntypes of *B. atlanticum*. The emended typification of this taxon is as follows:

Bupleurum atlanticum Murb. in *Lunds universitets årsskrift* Afd. 2, Sectio 2: 47-48 (1905) .

Type: Lectotype (selected here) – "*Bupleurum exaltatum* M. Bieb. var. *linearifolium* Boiss. Djebel Ksel près Géryville Sud de la prov. d'Oran, Algérie", 30.v.1856, Cosson (P!). Isolectotypes in G-BOIS!, K! & P! – several specimens in the latter.

Type locality: "L'intérieur du *Maroc* et de *l'Algérie occidentale*. [...]. *Mar.*: Dj Sidi-Fars, au sud de Maroc [...] – *Alg.*: Prov. d'Oran: Dj. Ksel près Géryville [...]; Dj. Taëlbouna près Asla [...]. Prov. d'Alg.: Dj. Tagga, sur le chemin de Boghar à Teniet-el-Haad; près de l'Oued Rhabat, env. Aumale [...]; Dj. Senalba, env. de Djelfa".

The specimen selected as lectotype of *B. atlanticum* clearly shows the typical stems with prominent 'verrucose' ridges as noted by Murbeck. The following syntype material was also revised: "Djebel Sidi-Fars au Sud de Maroc", 2.vii.1867, B. Balansa (P! – several specimens); "Djebel Taëlbouna sud de la province d'Oran", 11.v.1856, Cosson (P!); "Géryville (El Biob), sud de la province d'Oran", 27.v.1856, Cosson (P!); "Flora Gallia et Germania exsiccata de C. Billot. 2073 *Bupleurum montanum* Coss. [...] Pentes arides du Djebel Tagga, sur le chemin de Boghar à Teniet-el-Haad (Algérie)", 12.vii.1857, O. Debeaux (P! – several specimens).

22) *Bupleurum plantagineum* Desf., *Fl. atlant.* 1: 233-234 (1798).

Type: Holotype – "Herbier de la Flora Atlantique donné au Muséum, par M. Desfontaines - N°. *Bupleurum plantagineum*" (P-Desf.!).

Type locality: "Habitat in Atlante prope Bougie [Bejaïa, Algeria]."

Name origin: Maybe because its leaves are similar in shape to those of some species of *Plantago*.

Illustrations: Desf., *Fl. atlant.* 1 [plates]: tab. 57 (1798).

Shrub 60-160 cm tall, stems woody at the base, little branched, persistent after flowering. *Leaves* all similar, but upper leaves much smaller, (1-)2-16 x (0.2-)0.4-3(-4.2) cm, coriaceous, subamplexicaul, lanceolate to oblong-lanceolate, gradually attenuate to the base, acute to acuminate, rarely obtuse, mucronate, narrow marginal band smooth, parallel-veined, 5-14(-19) veins, visible, without secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal and lateral, terminal larger; *rays* (1-)4-15(-20), 0.4-3.4(-4.5) cm long, subequal, sometimes unequal, slender. *Bracts* 3-8, linear-lanceolate to lanceolate, erect-patent, much shorter than the rays, persistent in fruit. *Bracteoles* 5-7, 3-6 x 0.4-0.7 mm, subequal, lanceolate to linear-lanceolate, acuminate, longer and of similar width to the flowers or fruits. *Flowers* 5-11 per umbellule; *petals* yellow, with darker mid-vein, inflexed apex 2-lobed. *Fruits* clearly pedicellate, pedicels 3-6 mm long; mericarps 4-7.5 x 1-1.5 mm, oblong to oblong-elliptic, smooth; ridges narrowly winged and smooth.

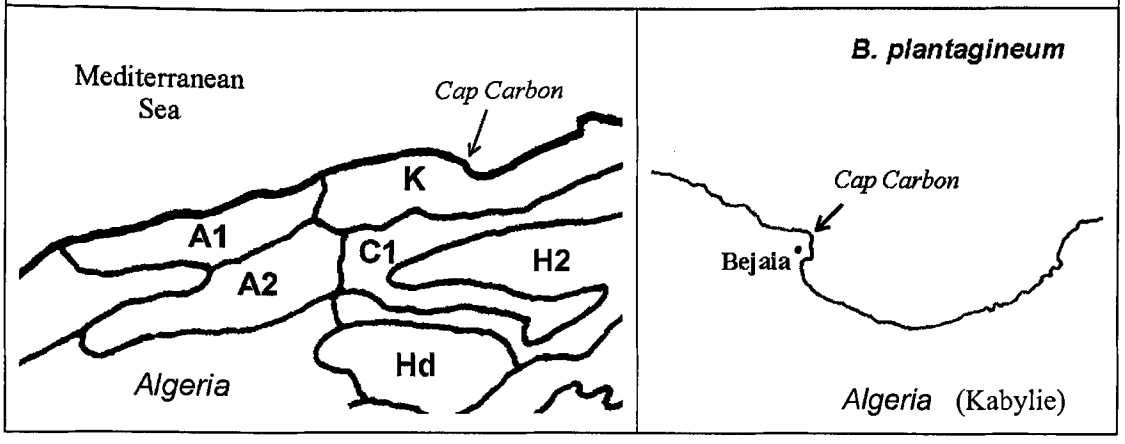
Chromosome Numbers: 2n = 28 (Cauwet, 1979a).

Ecology: Rocky cliffs, on limestone.

Altitude: 50-600 m. **Flowering time:** May – Sep.

World Distribution: Endemic to Algeria.

Distribution in NW Africa: *Algeria*: K (*Cap Carbon* area).



Representative specimens:

Algeria: K - Bougie [Bejaïa], 'rochers calcaires du Gouraya', vii.1898, E. Reverchon 41 (E; MA 86565; RNG); Alger [cultivated] "de semences récoltées au cap Carbon, près Bougie (loco unico classico), en novembre 1926" viii.1930, R. Maire (BC 141415; MA 86564 & 471292; RNG); Cap Carbon, near Bejaïa, 29.v.1971, Davis 52959 (BM, E, RNG); Béjaïa, "pentes E du Cap Carbon, 2 Km N de Béjaïa", 50 m, 1.vii.1982, A. Dubuis (MA 563168; MAF 149196), & ix.1982, H. Maurel & R. Rahmoun (MA 563167; MAF 149197).

Conservation status:

Bupleurum plantagineum has a very restricted distribution: it has only been recorded in the area of Cap [cape] Carbon in Algeria. There is no indication of how frequent is the plant in the area, but with such limited distribution the species is potentially endangered.

Critical taxonomic notes:

Bupleurum plantagineum can be mistaken for *B. canescens* Schousb., an endemic to S Morocco. These two shrubby species are morphologically close, but they differ in the shape and apex of leaves and bracteoles, and the length of the pedicels of flowers and fruits. *B. canescens* has oblong to oblong-lanceolate leaves which are always obtuse and mucronate-uncinate (with a very short hooked mucro); its bracteoles are ovate to ovate-lanceolate, acute; and pedicels are short [1-2.5(-3.5) mm]. In *B. plantagineum*, leaves are lanceolate to oblong-lanceolate, acute to acuminate, with a straight mucro; bracteoles are lanceolate to linear-lanceolate, acuminate; and pedicels are 3-6 mm long. Chromosome number is also distinct (2n = 32 in *B. canescens*).

Typification notes:

The first set of specimens for *Flora Atlantica* is kept as a separate herbarium in Paris (P-Desf.). There are duplicates of this material in Desfontaines own herbarium which was bought by Webb – see Steinberg, 1977, p. 5. Therefore, it is likely that there is an isotype of *B. plantagineum* Desf. in the herbarium of Webb (FI-W) in Florence (Firenze).

23) *Bupleurum salicifolium* R.Br. ex Buch, *Phys. Beschr. Canar. Ins.*: 195 (1825).

Type: Lectotype (selected here) – Thunberg herb.: "*Bupleurum salicifolium.* c. Madera. Masson" (UPS - IDC microfiche!) – see typification notes.

Type locality: No precise type locality, but the species was described from material of Madeira.

Synonyms: *Bupleurum salicifolium* Sol. ex Lowe in *Trans. Cambridge philos. Soc.* 6(3): 543 (1838); *B. aciphyllum* Webb ex Parl. in Webb & Berthel., *Hist. nat. Iles Canaries* [*Phyt. Canar.*] 2: 154 (1843).

Name origin: Because its leaves ('*folium*') resemble those of *Salix* spp.

Illustrations: Webb & Berthel., *Hist. nat. Iles Canaries* [*Phytogr. Canar.*]: tab. 70 (1838) [as *B. aciphyllum*]; M.A.Kunkel & G.Kunkel, *Fl. Gran Canaria* 4: 47, tab. 166 (1979); Bramwell & Z. Bramwell, *Flores Silv. Isl. Canarias* (2 ed.): 78, fig. 54 (1983); Press & M.Short, *Fl. Madeira*: 497, tab. 27, fig. 3 (1994).

Shrub 100-200 cm tall, stems becoming woody, ± branched, persistent after flowering. *Leaves* all similar, (1.2-)4-13(-15) x 0.3-1.8(-2.2) cm, generally herbaceous, sometimes coriaceous, subamplexicaul, linear-lanceolate to broadly lanceolate, gradually attenuate to the base, acuminate (often abruptly narrowing at the apex), often with uncinate apex, narrow marginal band smooth, parallel-veined,

5-10 veins, visible, sometimes with fine secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal and lateral, terminal larger; *rays* (1-)5-14(-20?), 0.6-4.7 cm long, generally subequal, slender to slightly thick. *Bracts* (2-)4-5(-6), linear-lanceolate to lanceolate, erect-patent to reflexed, shorter than the rays, persistent in fruit. *Bracteoles* 5(-6), 1-2.5 x 0.3-1.2 mm, subequal, linear-lanceolate to lanceolate, sometimes ovate-lanceolate, acute, much shorter and narrower than flowers or fruits. *Flowers* (3-)6-12 per umbellule; *petals* yellow, with darker mid-vein, or sometimes with 3 brownish veins, inflexed apical lobe entire. *Fruits* clearly pedicellate, pedicels 3-6 mm long; mericarps 5-7 x 1.2-1.6 mm, oblong-elliptic, smooth; ridges prominent to narrowly winged and smooth.

Chromosome Numbers: 2n = 32 (Cauwet, 1979a).

Ecology: Open vegetation or mixed forest; rocky places, on cliffs, on dry or humid habitats, in the open or in the shade; basaltic soils. Laurisylva; *Clethro-Laurion*.

Altitude: (100-)250-1300(-1650) m. **Flowering time:** (Apr.-)May – Oct.(-Nov.)

World Distribution: Endemic to Macaronesia.

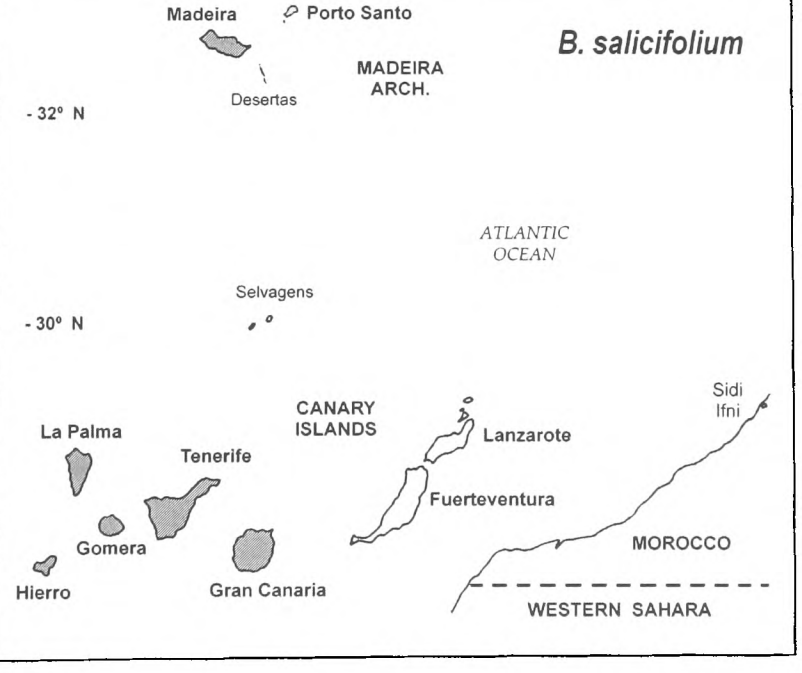
Distribution in Macaronesia:

Madeira Archipelago:

Madeira,
(Porto Santo).

Canary Islands:

Gran Canaria,
Gomera,
Hierro,
La Palma,
Tenerife.



Note: No material was seen for Porto Santo (Madeira Arch.), but there is a citation in the literature for this island: Pico Branco, 17.ix.1960, Rui Vieira 62 (MADJ – cited as 'BGF' or 'JBF') – see *Bol. Mus. Municip. Funchal* 24: 86 (1972); and *Bocagiana* 36: 33 (1974).

Vernacular names: anís silvestre, hinojo de risco (Spanish).

Representative specimens:

Canary Islands: *Gomera* - Roque de Cano de Vallehermoso, 30.vi.1969, D. Bramwell 2026 (RNG; SEV; & TFC 59); near Benchijigua, 9.v.1977, C.E. Jarvis (RNG); 'salida' [exit of] Tunel Hermigua, 29.vii.1977, M. Arco Aguilar *et al.* (TFC 25051); Cra. [road to] Valle Gran Rey, 16.vii.1974, W.W. *et al.* (TFC 4654). *Gran Canaria* - Roque Nublo, 27.iii.1969, D. Bramwell 1064 (RNG); between Ayacata and Tejeda, 19.iv.1973, W.T. Stearn 1163 (BM); Barranco de Guayadeque, c. Cuevas Muchas, 23.vi.1989, F. Amor (TFC 30688). El Sao, Agaete, 20.iv.1990, RMC (TFMC 2795). *Hierro* - 'Ile de Fer', [date?], C. Bolle s.n. (COI). *La Palma* - Bco. de las Angustias, 10.vi.1969, D. Bramwell 1895 (E; RNG; & TFC 60). *Tenerife* - 'in rupestribus convallis Guia', 20.vi.1855, H. de la Perraudière 1355 (COI - Herb. Willk.; E); Montañas de Teno, Bco. [Barranco] de Masca, 1.xii.1968, D. Bramwell 420 (RNG); Altos de Güimar, 9.ii.1989, M. Marrero *et al.* (TFC 29848); Bco. Chimaje, El Escobonal, 24.vii.1990, RMC (TFMC 2735). **Madeira Arch.:** *Madeira* - 'Curral dos Frieros' [Freiras], 12.vii.1865-1866, G. Mandon 121 (BM; COI - Herb. Willk.; E); Vale da Lapa, xi.1941, J.M. Carvalho (LISE); entre Caldeirões Verde e do Inferno, 27.vi.1951, Romariz 761 (LISU P43080); Serra de Agua, above Jardim da Serra, 24.x.1984, M.J. Short 145 (BM); Eira da Serra, 29.v.1989, LSP (TFMC 2000); between Pico do Arieiro and Pico do Ruivo, 29.xi.1989, L. Chilton & N.J. Turland (RNG).

Critical taxonomic notes:

1) Leopold von Buch validly published *Bupleurum salicifolium* in 1825, in 'his' "Verzeichniss der auf Madeira wildwachsenden Pflanzen". But this work was entirely based on Robert Brown's 'MS.' (= manuscript), including a list of the Madeira plants. The manuscript is kept at the Natural History Museum, London (Britten, 1904). Later, Lowe [*Trans. Cambridge philos. Soc.* 6(3): 543 (1838)] published a description of this species giving its authority to Solander. These two publications have generated some confusion about authority and valid publication of *B. salicifolium*. As Britten (1904, p. 3 & 41) explained, R. Brown's manuscript was largely based on that of Solander, and maybe Masson, and 'the descriptive phrase' of *B. salicifolium* was actually taken from Solander's 'MSS.', but the earliest publication of the species is undoubtedly that of Buch and the authority of R. Brown.

2) *Bupleurum salicifolium* has been subdivided into two subspecies: subsp. *salicifolium* (Madeira and Gomera) and subsp. *aciphyllum* (Canary Islands only) – see Cauwet & Sunding, 1981; Hansen & Sunding, 1993. Nevertheless, the only significant morphological difference between the 'subspecies' is the width of the

leaves. A peculiar aspect is that 'subsp. *salicifolium*' (with broader leaves) is believed to occur only in Madeira and Gomera (Canary Islands). Indeed, specimens with broader leaves are found in these islands, but I have also seen similar material from the islands of La Palma [Bco. del Agua de los Tilos, vii.1969, D. Bramwell (RNG)] and Hierro [Xinamar, 10.v.1899, R.P. Murray (BM); Jinama[r], P.L. Pérez *et al.* (TFC 4860)]. Furthermore, I have found material with narrower leaves from Madeira and Gomera – there is typical material of 'subsp. *aciphyllum*' in Gomera [La Fortaleza, near Chipude, 6.ix.1957, O.J. Gillie (E); Mirador Guadá, 13.iv.1981, Blás Méndez *et al.* (TFC 8884)]. Although maximum leaf width may be genetically determined, it is possible that the variation is enhanced by different ecological conditions. The habitats described for the specimens with broader leaves are often shady and humid, in contrast to dry and exposed for typical narrow leaved material – leaf texture also seems to be affected as only the exposed plants show more rigid leaves.

Cauwet & Sunding (1981) studied the anatomy of leaves and fruits from different populations of *B. salicifolium*. They did not find any differences in the anatomy of fruits. They found 3 types of leaf structure ('bifacial, sub-bifacial or subcentric'), but different types occurred in the same populations/islands without correlation with the proposed subspecific division. Apparently the only anatomical character that separated 'subsp. *salicifolium*' from 'subsp. *aciphyllum*' was the presence, in the latter, of an 'extra-ligneux' secretory canal (located externally to the xylem). But this is a small difference and a larger number of samples would have to be studied to confirm that this secretory canal is absent in all material from Madeira and Gomera (only 4 samples were studied for each of the islands).

Summarising, differences between populations of *B. salicifolium* are small and do not merit subspecific rank; maybe only varietal rank. Also, the geographical distribution of the typical material of the two 'subspecies' is not in agreement with a process of speciation – Madeira and Gomera islands are very distant from each other, making genetic exchange between their populations very difficult, so it is unlikely that they will evolve in the 'same direction'.

3) The sequences of the ITS region of *B. salicifolium* and *B. canescens* are only distinct by one or two base pairs (see chapter 9) suggesting that the two taxa are very closely related, maybe still at the species level with only the geographical separation

as the main obstacle of hybridization. Nevertheless, morphological differences (clear different shape of the leaves, and a much branched inflorescence in *B. salicifolium*) still seem enough to keep both taxa as distinct species.

Typification notes:

Neither the protologue of *B. salicifolium* or R. Brown's manuscript (see taxonomic note 1 above) provide information on type specimen or collector. Robert Brown's own collection of Madeira plants is likely to be small, as it was gathered in 1802 during only 4 days [see Britten, 1904, p. 3; or *Bocagiana* 51: 4 (1980)]. However, according to Britten (1904), R. Brown's list of Madeira plants seems to have been largely based on the collections of Francis Masson in this island, (1776-1778). Brown may have also used specimens collected by Banks and Solander, but they, too, spent onlya few days of 1768 collecting in Madeira.

Robert Brown's specimens can be found at BM, K and E, but there is no specimen of *B. salicifolium* collected by him in these herbaria. The main collections of Banks, Solander and Masson are also at BM; duplicates of Masson herbarium are in several herbaria. There is no material of *B. salicifolium* of these collectors at BM, nor at K or E. There is a specimen at BM collected by 'Masson' in 1857; unfortunately, this is not only a later date than the protologue but the collector is also a different person as Francis Masson died in 1805. There is a specimen at BM inside a type cover, but this is not type material as it is a Lowe specimen collected at a later date: July /47 [= 1847?] – Lowe was chaplain in Madeira between 1832-1854.

So far I have only found one specimen of the species collected by Masson in Madeira. It is in the Thunberg herbarium (UPS - IDC microfiche!). This specimen is undated, and the handwriting of the note "c. Madera. Masson" is that of Thunberg (cf. Burdet, 1978). Nevertheless, the Thunberg specimen is very likely a duplicate of the original material of *B. salicifolium*. As no original material was found at BM (K or E), which would have been preferred, the Thunberg specimen can, therefore, be chosen as lectotype.

24) *Bupleurum subspinosum* Maire & Weiller in *Bull. Soc. Hist. Nat. Afrique N.* 30: 19-20 (1940).

Type: Lectotype (selected here) – Herb. Maire: "In Atlantis Majoris monte Anremer: in glareosis calcareis supra lacum, 2700 m", 26.vii.1926, R. Maire (MPU!).

Type locality: [Morocco] "Hab. In lapidosis Atlantis Majoris: in monte Anremer supra lacum, solo calcareo, ad alt. 2700-2800 m [...]; in rupestribus schistaceis ditionis Reraya ad Tizi-n-Tamatert, ad alt. c. 2400 m [...]. In rupestribus Anti-Atlantis: in montium Sargho lapidocis vulcanicis ad Amalou-n-Ou-Mansour [...]; in lapidosis vulcanicis montium Siroua, ad alt. 2200-3300 m [...]."

Name origin: From the Latin '*sub*' (= somewhat, almost), and '*spinosus*' (= spiny), because this species resembles '*B. spinosum*', but the inflorescence is distinct and the rays, although stiff, do not look like spines.

Subshrub pulviniform (cushion-shaped) 20-30(-40) cm tall, stems becoming woody, much branched, persistent after flowering. *Leaves* all similar, 0.8-5.5 x 0.2-0.35 cm, ± herbaceous, ± amplexicaul, gradually attenuate to the base, lanceolate to oblong-lanceolate, acute, narrow marginal band smooth or sometimes minutely serrulate, parallel-veined, 3-5(-7) veins, visible, without secondary veins, thick intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal and lateral, lateral always 1-rayed, terminal with 1-3 *rays*, 0.2-0.6(-1.5) cm long, subequal, fine but becoming stiff. *Bracts* 1-2, ovate to lanceolate, erect-patent to appressed, shorter than the rays, persistent in fruit. *Bracteoles* 1-2(-3), 0.3-2 x 0.4-0.6 mm, subequal, ovate to lanceolate, acute to obtuse, shorter and narrower than flowers or fruits. *Flowers* 1-3(-4) per umbellule; *petals* yellow or yellow-greenish, sometimes with darker mid-vein, inflexed apex 2-lobed. *Fruits* sessile or subsessile, pedicels 0.1-1.5 mm long; mericarps 3-5 x 0.4-0.5 mm, oblong, smooth; ridges filiform and smooth.

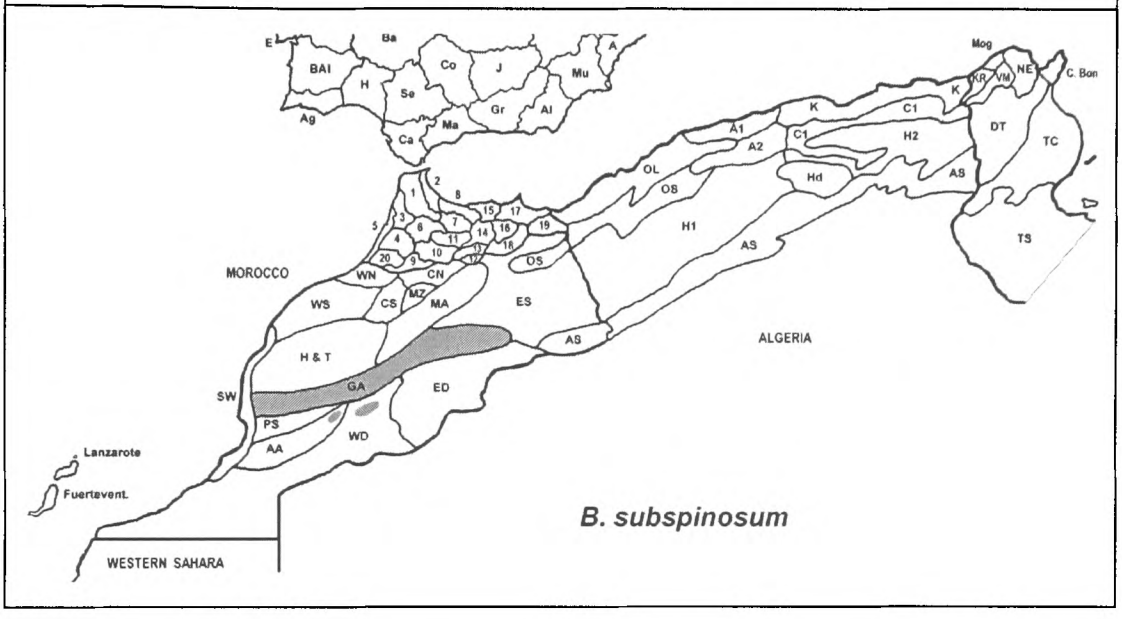
Chromosome Numbers: 2n = 32 (Cauwet, 1979a).

Ecology: High altitudes, in stony, rocky and dry places; slaty, calcareous or volcanic soils.

Altitude: 2100-3300 m. **Flowering time:** Jun. – Jul.

World Distribution: Endemic to Morocco.

Distribution in NW Africa: *Morocco*: AA (Jbel Siroua), GA, WD (Jbel Sarhro).



Type material revised:

Morocco: GA (*High Atlas*) - "In Atlantis Majoris monte Anremer [...], 26.vii.1926, R. Maire (MPU - Herb. Maire) [**lectotype**]"; "Tizi Tamatirt, 8000 ft, steep stony hillsides", 30.vi.1936, E.K. Balls B2988 (K) [**syntype**]. **WD** - "In rupestribus vulcanicis montium Sargho ad Amalou-n-Ou-Mansour, 2200 m", 22.vi.1939; Maire & Weiller 430 (K; MPU - Herb. Maire) [**syntypes**]. "Dj. Sargho: Amalou-n-Ou mansour", 2700 m, 23.vi.1939, Maire & Weiller 430 (MPU - Herb. Weiller) [**syntype**].

Other material found:

Morocco: GA - Oukaimedem, Jebel Angour, 15.vi.1974, Reading Univ./ BM Expedition 745 (BM; RNG); G.A.S. slope of Jebel Angour at about 10.000 ft, 21.vii.1976, C.J. & A.R. Humphries 99 (BM).

Conservation status:

I have only found 8 specimens of *B. subspinosum* amid all the herbarium material studied. The small number of specimens could mean that the species is very rare and might be endangered. However, it may have been under-collected because it can be mistaken for *B. fruticescens* subsp. *spinosum*, a much better-known plant and common in collections. Also, the fact that these few specimens came from localities that are fairly distant from each other, may indicate that *B. subspinosum*, although

apparently restricted to high altitude, is more common than we can now deduce. A detailed field survey is necessary to assess the true distribution and species vulnerabilty.

Critical taxonomic notes:

1) In the protologue, Maire & Weiller described *B. subspinosum* as having "*racemis umbellarum simplicibus*", i.e. that the species has 'simple umbels'. This description is incorrect because the inflorescence is indeed a compound umbel: we can see the bracts first, then 1 to 3 rays and then, before the umbellules with 1-3(-4) flowers, we have 1-3 small bracteoles. The lateral umbels are 1-rayed, but at least 1 bract and 1 bracteole are always present.

2) *B. subspinosum* can be mistaken for *B. fruticescens* subsp. *spinosum*, but the latter has normally 2-6 thick rays, tapering towards the tips, resembling spines after dispersal of fruits. At first sight, the inflorescence of *B. subspinosum* looks like an earlier stage of development of the inflorescence of '*B. spinosum*', but the trick is to note that what we see in *B. subspinosum* are not buds but developed flowers, and that lateral umbels are always 1-rayed.

25) *Bupleurum angulosum* L., *Sp. pl.*: 236 (1753).

Type: Lectotype (selected here) – Herb. Burser 16: 4 (UPS - IDC microfiche!).
Type locality: "Habitat in Pyrenaeis."

Synonyms: *Bupleurum pyrenaeum* Gouan, *Ill. observ. bot.*: 8, tab. 4 (1773); "*B. pyrenaicum*" auct. non Gouan.

Name origin: In the protologue, Linnaeus described the leaves as angular ('*folio anguloso*'), but he was only following earlier phrase names [e.g. C. Bauhin's (1623) '*Perfoliata alpina angustifolia major, sive folio anguloso*']. This is a strange characterisation of the leaves of this species, maybe only meaning that leaves spread at 'particular [but not unusual] angles'. However, one could attribute the epithet to the fruits: they are indeed 'angled', as the ridges are very prominent.

Illustrations: H.Wolff in Engl., *Pflanzenr.* 43 (IV.228): 58, fig. 8 (1910); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 441 (1990).

Perennial herb 15-70 cm tall, stems herbaceous, little branched, withering after flowering. *Leaves* herbaceous, amplexicaul, acute to obtuse, narrow marginal band smooth, pinnate, with thick midrib and a reticulum of fine lateral veins, intramarginal vein absent; *basal leaves* 4-30(-40) x 0.3-3 cm, gradually attenuate to the base into petiole, linear-lanceolate to oblong-lanceolate, persistent during flowering, petiole very short or up to 1/2 of total length of the leaf; *cauline leaves* sparse and differing in shape and insertion to the basal leaves, 1.5-9.5(-25) x 0.8-1.5(-2) cm, sessile, cordate-amplexicaul, ovate-lanceolate. *Umbels* terminal and lateral, the latter smaller; *rays* 3-6(-8), 1-5(-7) cm long, subequal or unequal, ± thick but flexible. *Bracts* (2-)3-5, ovate to elliptic, erect-patent or patent, shorter than the rays, persistent in fruit. *Bracteoles* (4-)5-6, 5-20 x 4-15 mm, subequal, suborbicular to oblong, obtuse, longer and broader than flowers or fruits. *Flowers* 10-40(-50) per umbellule; *petals* yellow, sometimes greenish or purplish, without darker mid-vein, inflexed apical lobe entire. *Fruits* clearly pedicellate, pedicels 3-6 mm long; mericarps 4-7 X 0.8-2.5 mm, oblong to oblong-elliptic, smooth; ridges narrowly winged and smooth.

Chromosome Numbers: 2n = 14 (Cauwet, 1979a).

Ecology: Pasture and rocky places in hills and mountains, in the shade or in the open; on limestone, conglomerate or granite, acid soils. Woods of *Pinus nigra* or *Betula* sp.; *Saxifragion mediae.*

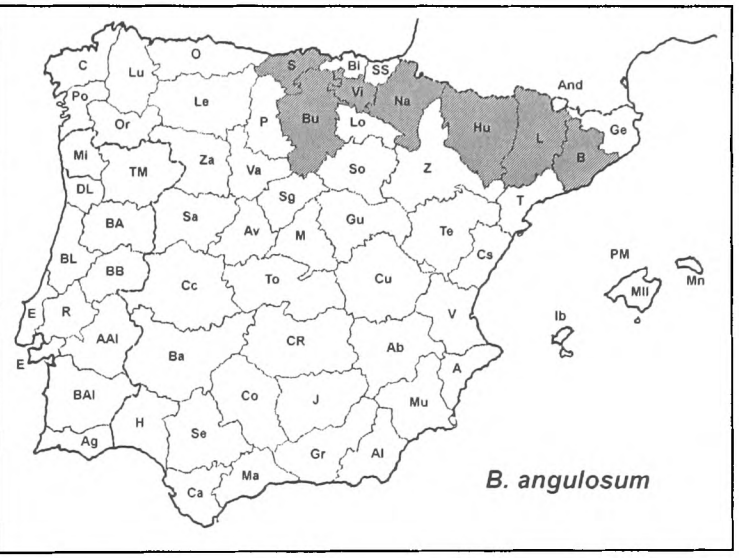
Altitude: 550-2450 m. **Flowering time:** May – Aug.(-Sep.)

World Distribution: Endemic to the Pyrenees and mountains of N Spain.

Distribution in the Iberian Peninsula:

Spain:

B, Bu, Hu, L, Na, S, Vi.



Notes: There is a citation of *B. angulosum* for the province of Castellón (Peñagolosa) [*Anales Jard. Bot. Madrid* 12(1): 487 (1953)], but the plant referred to is undoubtedly *B. ranunculoides* – I have also seen herbarium material from the locality.

Vernacular names: ilebrenca angulosa (Catalán).

Representative specimens:

Spain: Alava (Vi) - Sierra de Cantabria, vii.1934, Losa (MA 417892); Santacruz de Kampezo, 21.viii.1983, B. Betoño *et al.* (MA 417892); Barcelona - Montserrat, vii.1910, Font Quer (MA 86194); Berga, vii-viii.1911, F. Sennen (MA 86197); Burgos - Espinosa de los Monteros, 3.viii.1995, Moreno Moral *et al.* (MA 564992); Cantabria (S) - Portillo de Lunada, 20.viii.1970, Pereda Saez (MA 423412); Portillo de la Silla, vi.1980, T.E. Diaz González (MGC 8929); Soba, 9.viii.1995, Aedo *et al.* (MA 561252); Huesca - Castejón de Sos, 5.viii.1995, V.J Arán & Tohá (MA 561279); Balneario de Panticosa, 15.viii.1981, M. Fernández-Carvajal (MA 565295); Torla, 14.viii.1984, M. Luceño (MA 423981); Lérida - Valle de Arán [date?], Villiers (MA 86192); Bonaigüa, 22.vii.1975, E. Valdés *et al.* (MA 486224); Lago de Cavallers, 10.viii.1980, F. Llamas (MGC 8952); Navarra - Cabredo, 27.viii.1989, B. Betoño & J. Alejandre (MA 486224); Isaba, 5.viii.1987, P.M. Uribe-Echebarría (MA 478680).

Critical taxonomic notes:

1) Occasionally, material of *B. angulosum* has been wrongly identified as *B. ranunculoides*. Indeed, even the Linnaeus concept of *B. angulosum* included part of the material we now regard as *B. ranunculoides* (see typification notes below). His variety 'β' of '*B. angulosum*', occurring in the Alps ("β. in Vallesiae alpibus"), corresponds to material of *B. ranunculoides* with cauline leaves broadly cordate-amplexicaul, material more common in C Europe (see taxonomic notes of *B. ranunculoides*). However, *B. ranunculoides* and *B. angulosum* are easily distinguished by the venation of the lower leaves: parallel in the former and pinnate-reticulate in the latter.

2) *Bupleurum angulosum* may also be mistaken for the closely related *B. stellatum* (endemic to the Alps). The two species are morphologically very similar, but easily distinguished by their involucel: bracteoles free in *B. angulosum*, and fused (connate) at least in 2/3 of the total length in *B. stellatum*.

Typification notes:

The following is the original material of *Bupleurum angulosum* (two syntypes cited in the protologue): Herb. Burser 16: 4 (UPS - IDC microfiche!); Herb. Burser 16: 6 [referring to var. 'β'] (UPS - IDC microfiche!). There is a specimen in the Linnaeus herbarium at UPS annotated "*Bupleurum angulosum* Ard.", but because it lacks the *Species Plantarum* number it cannot be regarded as original material. However, Burser's specimen 16: 6 is not *B. angulosum*, but *B. ranunculoides*. Therefore, the only choice of type is Burser's 16: 4, that is indeed a good specimen of *B. angulosum*.

26) *Bupleurum foliosum* Salzm. ex DC., *Prodr.* 4: 133 (1830).

Type: Lectotype (selected here) – Herb. DC.: '*Bupleurum foliosum* mihi', Tanger, 1825, Salzmann (G-DC – IDC microfiche!).

Type locality: "in Mauritaniâ circà Tanger".

Synonyms: *Bupleurum obliquatum* Schousb. ex Ball in *J. Linn. Soc., Bot.* 16: 466 (1878) – pro syn., nom. inval.; *B. tortuosum* Schousb. ex Ball in *J. Linn. Soc., Bot.* 16: 466 (1878) – pro syn., nom. inval. [see *Code* Art. 34.1(c)].

Name origin: From the Latin '*foliosus*' (= leafy); maybe because of the shorter internodes of the lower leaves (upper leaves are sparse).

Illustrations: H.Wolff in Engl., *Pflanzenr.* 43 (IV.228): 167, fig. 20A-C (1910); Valdés *et al.* (eds), *Fl. Andalucía Occid.* 2: 313 (1987).

Subshrub 20-75 cm tall, stems becoming woody, little branched, persistent after flowering. *Leaves* (1-)3-10 x 0.2-1.2 cm, ± coriaceous, subamplexicaul, acute to acuminate, narrow marginal band smooth, pinnate, with ± thick midrib and a reticulum of fine lateral veins, intramarginal vein absent; *basal leaves* withering before flowering; *lower leaves* sometimes crowded or tufted, sessile or slightly attenuate to the base, linear-lanceolate to narrowly oblong-lanceolate; *upper leaves* sparse, differing in shape and insertion to lower, narrowly cordate-amplexicaul, ovate to lanceolate. *Umbels* terminal and lateral, all similar; *rays* 1-3(-4), 0.6-4 cm long, subequal, slender. *Bracts* 1-3, ovate, erect-patent, shorter than the rays, persistent in fruit. *Bracteoles* 5-6, 4-6 x 1-3 mm, subequal, ovate to ovate-lanceolate, obtuse-mucronate, of similar length but broader than flowers or fruits. *Flowers* 3-15 per umbellule; *petals* yellow or greenish-yellow, sometimes with darker mid-vein, inflexed apical lobe entire. *Fruits* pedicellate, pedicels 1.5-5 mm long; mericarps 4-6 x 1-1.5 mm, oblong-elliptic, smooth; ridges narrowly winged and smooth.

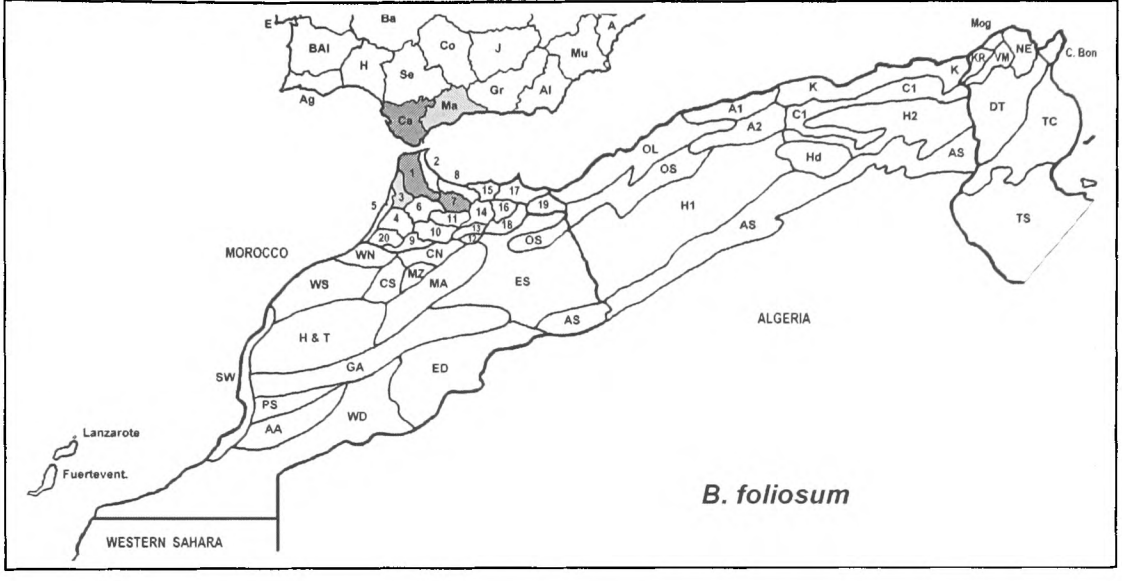
Chromosome Numbers: 2n = 14 (Cauwet, 1979a).

Ecology: Mediterranean open vegetation, hills or mountains; on sandstone and non calcareous soil, rarely on serpentine soil. *Ericetum, Cistetum.*

Altitude: 200-1250(-1400) m. **Flowering time:** Jun. – Oct.

World Distribution: Endemic to the W Mediterranean: SW Spain and N Morocco.

W Mediterranean Distribution: *Spain*: Ca, Ma. *Morocco*: 1, 3?, 7.



Notes: There is only one specimen known for the province of Málaga (MGC 23807 - see below). Although no material was seen, a specimen is cited for Larache (Morocco - region 3) – Jahandiez & Maire (1932, p. 532).

Representative specimens:

Spain: Cádiz: Sierra de Palma, près Algeciras, 17.vii.1887, E. Reverchon 23 (E); Sierra de Algeciras, 20.vi.1988, B. Cabezudo *et al.* (MGC 22525); Puerto de Galiz, 9.vii.1983, J. Arroyo (SEV 119615); Cerro del Puerto de Hoyo, 21.x.1970, B. Molesworth-Allen (SEV 7585). Málaga: Estepona, Peños Blancos, 14.vii.1988, B. Cabezudo *et al.* (MGC 23807) – only known specimen for the province. **Morocco: 1** - Tangier, 1803, Durand 28 - Herb. Smith 485.24 (LINN); "In ericetis tingitanis", 17.viii.1831, Salzmann (MPU); Dj. Quebir, pr. Tandja [Tanger], 8.vii.1930, Font Quer (BM; MA 86765); **7** - Near Bab Berret, 6.vii.1973, Davis 54908 (E); Bab "Beret" to Bab Besen, 17.viii.1970, Davis 50671 (BM, RNG).

Conservation status:

A species with a very restricted distribution and unknown vulnerability status, requiring detailed field survey.

Critical taxonomic notes:

Bupleurum foliosum may be confused with some specimens of *B. gibraltarium* (q.v.), but this closely related species is more robust, has numerous rays, (3-)5-25 (-57), and reflexed bracts.

Typification notes:

Although there are other Salzmann specimens collected in Tanger, with the annotation '*Bupleurum foliosum* mihi' (two in E! & another two in K!), the specimen in De Candolle's herbarium (G-DC!) is almost certainly the one used by the author to describe the new species, and is therefore selected here as lectotype. As these specimens are very poorly annotated, we cannot be absolutely sure that they all belong to a single collection and, therefore, the specimens at E and K are here considered only syntypes.

27) ***Bupleurum fruticosum*** L., *Sp. pl.*: 238 (1753).

Type: Lectotype (selected here) – Herb. Clifford 104, *Bupleurum* 1 (BM!).
Type locality: "Habitat in Galliae australis saxosis maritimis."

Name origin: From the Latin '*fruticosus*', meaning shrubby.

Illustrations: H.Wolff in Engl., *Pflanzenr.* 43 (IV.228): 167, fig. 20D-J; 169, fig. 21 A-C (1910); Tutin, *Umbellifers Brit. Isl.*: 123 (1980); Valdés *et al.* (eds), *Fl. Andalucía Occid.* 2: 314 (1987); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 442 (1990).

Shrub 60-200(-300) cm tall, stems becoming woody, ± branched, persistent after flowering. *Leaves* all similar, (1-)3-13 x (0.4-)1-4.5 cm, coriaceous, subsessile, gradually attenuate to the base, with a very short petiole, oblong-lanceolate to oblong-elliptic, more rarely obovate, acute to obtuse, shortly mucronate, narrow marginal band smooth, pinnate, with thick midrib and a reticulum of fine lateral veins, intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal (very exceptionally with a few lateral umbels); *rays* (3-)6-20(-25), 1-6 cm long, subequal, thick. *Bracts* 5-8, oblong to obovate, spreading, shorter than the rays, falling during fruiting (deciduous). *Bracteoles* 4-6, 2.5-8 x 1-3 mm, subequal or unequal, linear-lanceolate to oblong-lanceolate, mucronate, longer and broader than flowers or fruits. *Flowers* (5-)7-18 per umbellule; *petals* yellow, sometimes with darker mid-vein, inflexed apical lobe entire. *Fruits* clearly pedicellate, pedicels (3-)4-12 mm long; mericarps 5-7(-8) x 1-1.5 mm, oblong to oblong-elliptic, smooth; ridges narrowly winged and smooth.

Chromosome Numbers: 2n = 14 (Cauwet, 1979a).

Ecology: Mediterranean woods and maquis, normally in the shade, sometimes near streams or drainage gullies; on calcareous, dolomitic, siliceous or slaty substratum; acid soils. Oak and pine woods; *Querceto-Juniperetum*, *Subereto-Quercetum*, *Quercetum ilicis*.

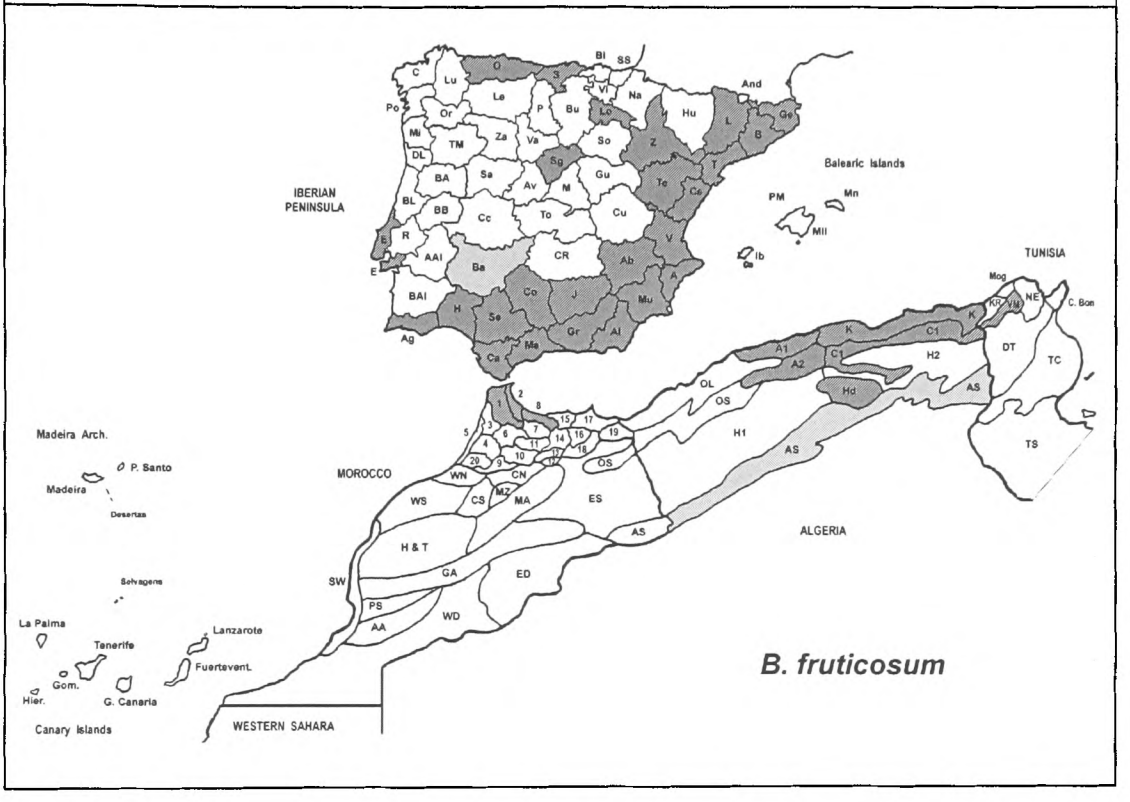
Altitude: 0-1200(-1975) m. **Flowering time:** May – Oct.(-Nov.).

World Distribution: Mediterranean region; from the Iberian Peninsula to Greece,

and NW Africa (for more detailed distribution see Browicz, 1982; and also Siddiqi, 1986).

W Mediterranean Distribution:

Spain: A, Ab, Al, B, (Ba), Ca, Co, Cs, Ge, Gr, H, J, L, Lo, Ma, Mu, O, S, Se, Sg, T, Te, V, Z. *Portugal*: Ag, E. *Morocco*: 1, 2, 8. *Algeria*: A1, A2, AS, C1, Hd, K. *Tunisia*: VM.



Notes: Although no material was studied from the province of Badajoz, there is a reference to *B. fruticosum* for Sierras de Alconera y de los Santos, and Jerez de los Caballeros [*Anales Jard. Bot. Madrid* 17(2): 364 (1959)].

Vernacular names: douag (Arabic); matabou (Catalán); shrubby hare's-ear (English); beleza (Portuguese); adelfilla, balabre, batabuey, beleza, bupleiro, clujía fina, costibuey, costilla de buey, cuchillerela, matabuey, reores (Spanish).

Representative specimens:

Spain: Albacete, Yeste, 15.viii.1985, C. Soriano (MA 462572); Almería, Sierra de Gador, 29.vii.1992, A. Hervás (GDA 37510); Asturias, Oviedo, Grado, 29.viii.1978, E. Rico (MA 310475); Barcelona, Montserrat, 6.viii.1916, Font Quer (MA 86737); Cádiz, Grazalema, Los Batanes, 11.ix.1973, S. Silvestre & B. Valdés (MA 504381); Castellón, Lucena del Cid, 11.vi.1933, H. del Villar (MAF 57692); Gerona, Costa Brava, San Grau, 5.xi.1973, H. Kuhbier (SEV 20401); Granada, Dílar, 22.viii.1990, F. Castilla & R. Gamarra (MA 488363); Huelva, Sierra de Aracena, 30.viii.1975, Pérez-Chiscano (MA 339975); Jaén, Beas de Segura, 1.v.1985, C. Soriano (MA 462129); Málaga, Sierra de Tejeda, Alcaucín, 26.vi.1982, J. Nieto (MA 316171); Tarragona, Conca de Barbera, 23.viii.1990, J. Pedrol (MA 509464); Zaragoza, La Cartuja, 15.viii.1992, N. Mercadal (JACA

377294). **Portugal:** Algarve, Serra de Monchique, Meia Viana, 9.viii.1997, S. Neves 30A (E); Estremadura - V. Nogueira de Azeitão, 24.vii.1962, P. Silva & J. Martins 7897 (COI); Sesimbra, 28.ix.1973, A. Matos & M. Alves 12751 (COI). **Morocco: 2** - Tetuan [date?], Broussonet 49 (MA 86749); **8** - Targuist, 1 & 17.vii.1927, Font Quer (BC 25718 & MA 86751). **Algeria: K** - Chabet el Akra Gorge, below Kherrata, 23.v.1971, Davis 52692 (RNG); 50 km of Jijel, 5 km of Ziama Mansouria, 28.iv.1976, D.A. & S.J. Sutton 880 (RNG).

Critical taxonomic notes:

1) *B. fruticosum* might be confused with *B. gibraltarium*, but the latter always has lateral umbels, and its leaves are glaucous on both faces (unifacial), while leaves of *B. fruticosum* are green above and glaucous underneath.

2) Rarely some specimens of *B. fruticosum* have both terminal and lateral umbels, but otherwise are identical to the typical material, e.g.: S Portugal, Monchique - S. Neves 28 (MA) and S. Neves 30A (E).

Typification notes:

The following is the original material of *B. fruticosum*: Herb. Burser 8: 10 (UPS - IDC microfiche!); Herb. Clifford 104, *Bupleurum* 1 (BM!); Herb. Linn. 335.26 (LINN!); [icon!] '*Seseli aethiopicum frutex*' in Dodoens, *Hist. stirp. pempt.* ed. 2: 312 (1616). The specimen in Clifford's herbarium is chosen here as lectotype because it is more complete and in better condition than the specimens at LINN and UPS.

28) *Bupleurum gibraltarium* Lam., *Encycl.* 1: 520 (1785).

Type: Lectotype (selected here) – Herb. Lam.: "*Bupleurum* ... de gibraltar: tige de 4 pieds, ligneuse, droite, presque simple ou garnie de q[uel]ques rameaux alternes et mediocres à son sommet, feuilles longues etc. fl[eures] jaunes; involucres reflechis sur les pedoncules etc." (P-LA!).

Type locality: "Ce Buplèvre croît aux environs de Gibraltar: on le cultive au Jardin du Roi."

Synonyms: *Bupleurum coriaceum* L'Hér., *Stirp. nov.*: 139, tab. 67 (1791); *B. verticale* Ortega ex Lange in Willk. & Lange, *Prodr. fl. hispan.* 3: 76 (1874).

Name origin: From the type locality, cultivated material in Gibraltar. Supposedly, the species "grows around Gibraltar"; unfortunately, 'around' has to be viewed in a very broad sense: *B. gibraltarium* does not spontaneously occur in Gibraltar, but does indeed in the relatively close Spanish mainland.

Illustrations: Pott.-Alap., *Fl. Tunisie* 1: 586, fig. 924 (1979); Valdés *et al.* (eds), *Fl. Andalucía Occid.* 2: 313 (1987); O.Bolòs & Vigo, *Fl. Països Catal.* 2: 442 (1990).

Shrub 60-150 (-200) cm tall, stems becoming woody, ± branched, persistent after flowering. *Leaves* all similar, 3-24 x 0.3-3 cm, coriaceous, subamplexicaul, gradually attennuate to the base, sometimes with a short petiole, lanceolate or narrow to broadly oblong-lanceolate, acute, mucronate-uncinate, narrow marginal band smooth or minutely crenulate, pinnate, with thick midrib and a reticulum of fine lateral veins, intramarginal vein absent; *basal leaves* withering before flowering. *Umbels* terminal and lateral, the latter smaller; *rays* (3-)5-25(-57), 1-10(-13) cm long, subequal, rarely unequal, ± thick. *Bracts* (3-)4-7(-12), ovate to lanceolate, reflexed, much shorter than rays, persistent in fruit. *Bracteoles* (3-)5-6(-7), 2-9 x 2-3(-5) mm, subequal, sometimes unequal, ovate-lanceolate, acute, longer and broader than flowers or fruits. *Flowers* (3-)5-10(-25) per umbellule; *petals* yellow or greenish-yellow, sometimes with darker mid-vein, inflexed apical lobe entire. *Fruits* clearly pedicellate, pedicels 2-7(-11) mm long; mericarps 4-8(-11) x 1-2 mm, oblong to oblong-elliptic, smooth; ridges narrowly winged, smooth.

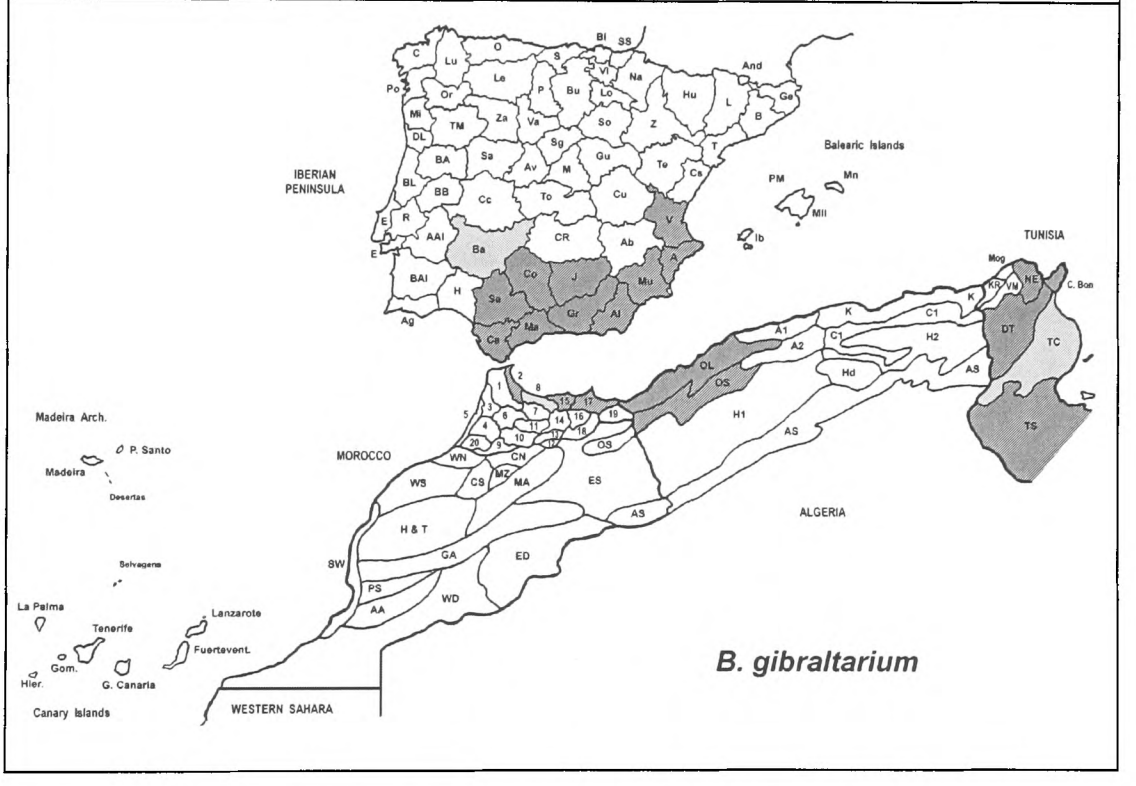
Chromosome Numbers: 2n = 14 (Cauwet, 1979a).

Ecology: Mediterranean open vegetation, rocky slopes or cliffs, often exposed; on limestone, marl or dolomite, sometimes on schist. *Quercetalia ilicis*, *Oleo-Ceratonion*, *Bupleuro-Ononidetum speciosae*.

Altitude: 0-1500 m. **Flowering time:** Jun. – Oct.(-Nov.).

World Distribution: Endemic to the W Mediterranean: S & C Spain & NW Africa.

W Mediterranean Distribution:
Spain: A, Al, Ba, Ca, Co, Gr, J, Ma, Mu, Se, V. *Morocco*: 2, 8?, 15, 17. *Algeria*: OL, OS. *Tunisia*: C. Bon, DT, NE, TC?, TS.



Note: Only one undated specimen has been found for the province of Badajoz [Mérida, vii., M. Rivas Mateos (MAF 53051)]; the species is also indicated for this area in the distribution map in Bolòs & Vigo (1990), but seems to be very rare. There is an incomplete specimen of *B. gibraltarium* reputedly from Navarra in the Spanish Pyrenees [c. Orbaiceta, Peña de Estaboa, vi-vii.1786, L. Nee (MA 86493)]. However, there is no other reference for the occurrence of the species in the area, and, therefore, it is likely that the specimen has been mislabelled.

Vernacular names: cluigida (Catalán); colleja, crujía, cuchilleja (Spanish).

Representative specimens:

Spain: Alicante, Campoamor, S. Miguel de Salinas, 16.v.1965, A. Rigual (MA 369538); Almería, Vicar-Enix, km 1, 9.xi.1969, J.F. Casas (MA 415052); Cádiz, Benahoma, 8.ix.1983, A. Aparicio & S. Silvestre (MA 469041); Córdoba, Sierra Halconera, Priego, vi.1960, J. Borja (MAF 69784); Granada, Almejigar, 4.x.1980, Ladero & Molero (MA 346342); Sevilla, c. Coripe, Rio Guadalporcún, 15.viii.1997, S. Neves 34 (MA); Valencia, Mogente, Barranco del Agua, v. 1980, M. Costa *et al.* (MAF 111825). **Morocco: 2** - J. Tisouka, above Xanen, 5.vii.1973, Davis 54807 (E); **17** - Melilla, Cabo Tres Forcas, 28.viii.1930, Hno. Mauricio (BC 141404, MA 86700). **Algeria: OL** - Oran, Santa Cruz, ix.1930. A. Faure (BC 141406, RNG). **Tunisia: TC**?- Fériana, Djebel Go[...?], 02.viii.1884, Robert [?] Herb. Giraudias (MA 86703).

Critical taxonomic notes:

1) This species might be confused with the closely allied *B. foliosum* and *B. fruticosum*. *B. gibraltarium* is generally more robust and taller than *B. foliosum*, but the latter is easily distinguished by its short number of rays (1-3), erect to patent bracts (never reflexed), cordate-amplexicaul upper leaves and divaricate flowering stems, these all similar in length, in contrast with *B. gibraltarium* ± straight, main flowering stem with shorter lateral branches. *B. fruticosum* is easily recognized because it has only terminal umbels (only exceptionally lateral are present), and its bifacial leaves: green above, and glaucous underneath, while those from *B. gibraltarium* are glaucous on both surfaces.

2) A few specimens of *B. gibraltarium* have non typical inflorescences for the genus, where what should have been pedicels, become rays of small umbels of third order [e.g.: Alicante, Campoamor, A. Rigual (MA 369567)]. Also, but rarely, some specimens have single flowers appearing between the rays of the umbel [e.g.: Almería, Cabo de Gata, F. Fábregas (MA 86493)].

Typification notes:

There are two specimens in Lamarck's herbarium (P-LA!), but only one can be regarded as original material; this specimen has a short description written by Lamarck [cf. his handwriting in *Candollea* 31: 151-152 (1976)] with information also used in the protologue. The second specimen ["*Bupleurum foliis verticalibus* prope Algeris. Pourret" (P-LA!)] is not from the type locality as it was collected in NW Africa (near Alger?). Isolectotypes are not known.

29) *Bupleurum rigidum* L., *Sp. pl.*: 238 (1753).

Type: Lectotype – Herb. Linn. 335.19 (LINN!) [selected by Reduron, J.-P. & Jarvis, C. – in preparation].

Type locality: "Habitat Montpellier." (S France).

Name origin: From the Latin '*rigidus*' (= rigid, hard), because of its leathery, hard textured leaves.

Perennial herb (12-)30-150 cm tall, woody at the base, stems herbaceous, often very branched, sometimes diffusely branched, withering after flowering. *Leaves* all similar or the basal different in shape and insertion, all leaves (1-)10-45 x 0.1-6.5 cm, coriaceous, ± amplexicaul, obtuse to acuminate, narrow m7arginal band smooth or minutely crenulate, ± parallel-veined, (1-)3-13 veins, all prominent, sometimes with thick secondary lateral veins, thick intramarginal vein present and generally as prominent as the midrib (especially in the lower leaves); *basal leaves* ± crowded, linear to oblong-lanceolate, sometimes obovate or spatulate, leaf blade sometimes rapidly (or gradually) attenuating into petiole, basal leaves withering but persistent during flowering; *cauline leaves* sparse, sessile, linear or linear-lanceolate. *Umbels* terminal and lateral, all similar; *rays* (1-)2-6, (0.3-)1-6(-9) cm long, subequal, slender. *Bracts* 1-5, linear, appressed to erect-patent, much shorter than the rays, present during fruiting. *Bracteoles* (2-)4-5(-7), 1-1.5 x 0.2-0.4 mm, subequal, linear, acuminate, shorter and narrower or similar in size to flowers or fruits. *Flowers* (1-)3-10(-12) per umbellule; *petals* yellow or yellow-greenish, sometimes with darker mid-vein, inflexed apical lobe entire. *Fruits* generally long pedicellate, pedicels (1-)3-8(-11) mm long; mericarps 3-6(-7) x 1-2 mm, oblong to oblong-elliptic, smooth; ridges filiform and smooth.

Chromosome Numbers: 2n = 14, 16 (Cauwet, 1979a).

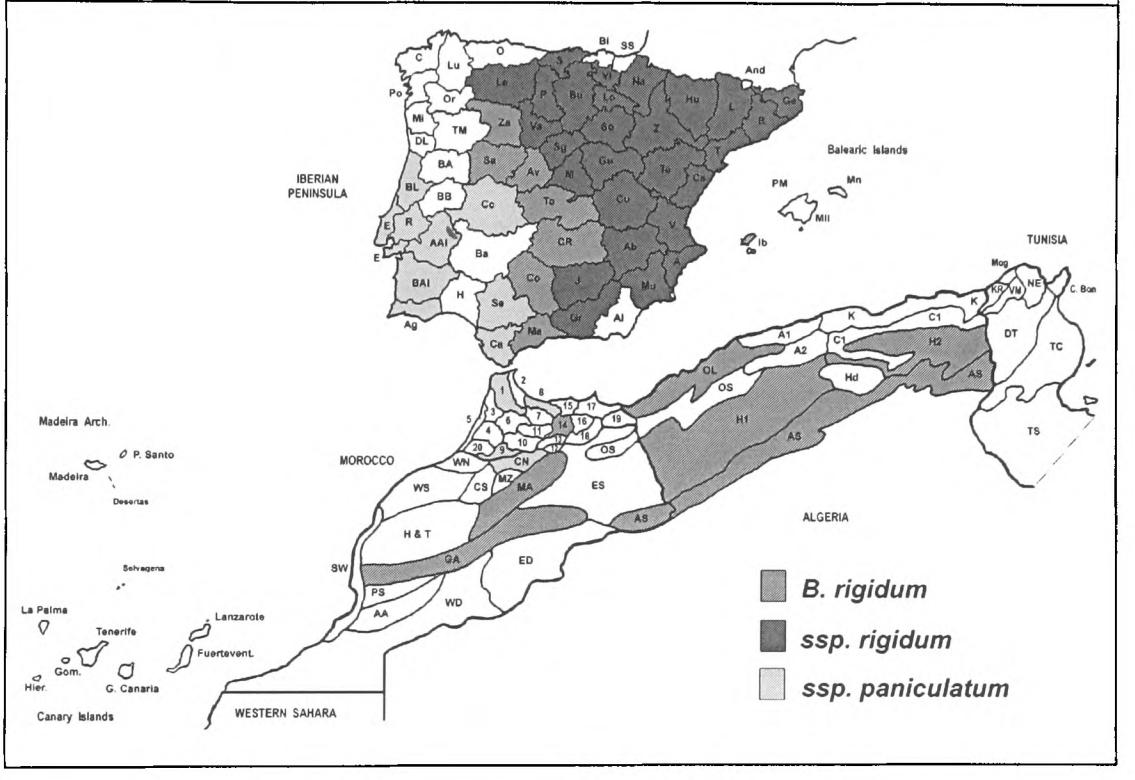
Ecology: Mediterranean vegetation, maquis, woods, sometimes pasture, in the open or in the shade; generally on calcareous soil, sometimes siliceous, on gypseous marl, dolomite, sandstone or clay, dry soils. *Aphylanthion, Brachypodion phoenicoidis, Quercion ilicis, Quercion pubescenti-petraeale, Querco-Lentiscetum*, etc.

Altitude: 0-1500(-1900) m. **Flowering time:** (Apr.-)Jun. – Oct.(-Nov.).

World Distribution: W Mediterranean - Iberian Peninsula, S France & N Italy, Morocco & Algeria.

W Mediterranean Distribution:

Spain: A, Ab, Av, B, Bu, Ca, Cc, Co, CR, Cs, Cu, Ge, Gr, Gu, Hu, J, L, Le, Lo, M, Ma, Mu, Na, P, (PM [Ib]) S, Sa, Se, Sg, So, T, Te, To, V, Va, Vi, Z, Za. *Portugal*: AAl, Ag, BAl, BL, E, R. *Morocco*: 1, 8, 9, 14. AS, CN, GA, MA. *Algeria*: AS, H1, H2, OL.



Notes: The areas of the map (above) attributed to *B. rigidum* (s.l.) correspond to provinces or regions where: **a)** the material studied is intermediate between the two subspecies; or **b)** typical material of both subspecies is found. In the case of Algeria, all the area is treated as of *B. rigidum* s.l. because there I am not certain about the distribution of the two subspecies or if both do indeed occur. It seems that all the material in Algeria corresponds to *B. rigidum* subsp. *rigidum* (see description of Quézel & Santa, 1963). The few Algerian specimens I found were indeed of that subspecies, but they were all from the same locality/area, and therefore not a representative sample.

Critical taxonomic notes:

Bupleurum rigidum is easily recognizable because of its hard-textured leaves, with very prominent veins. In particular, the intramarginal veins are generally as prominent as the thick midrib [the intramarginal vein is the vein that runs parallel to the border of the leaf, immediately inside or very near to the narrow marginal band]. Also, the basal leaves of *B. rigidum* are always very long (normally around 20-30 cm long, but they can be as long as 45 cm! – almost certainly the longest in the genus).

Typification notes:

The following is the original material of *B. rigidum*: Herb. Burser 16: 9 (UPS - IDC microfiche!) [this specimen is basically destroyed as only the fruits still remain in the herbarium sheet]; Herb. Linn. 335.19 (LINN!); & Herb. Linn. 335.20 (LINN!). However, the sheet 335.20 in LINN does not contain material of *Bupleurum*, in fact the specimen is not even an *Umbelliferae* (maybe *Rubiaceae*?). This specimen is original material because it has the number 9 corresponding to *B. rigidum* in *Species Plantarum*, and indication of the type locality ('Monsp.' = Montpellier); the specimen is also pinned to sheet 335.19 that is, indeed, a good specimen of *B. rigidum* – the chosen lectotype. However, I do not believe that Linnaeus could have ever confused this specimen with *B. rigidum*, so the only explanation is that the specimen has been misplaced. Therefore, the specimen chosen as lectotype is the only possible choice.

Key to identification of subspecies:

1a – Basal leaves abruptly attenuate into petiole, leaf blade 0.7-6.5 cm broad, obovate to oblong-lanceolate or spatulate, 5-13 main veins, with numerous secondary veins. ..... **a.** subsp. ***rigidum***.

1b – Basal leaves sessile, no or gradually attenuate to the base, 0.5-1.2 cm broad, linear to linear-lanceolate, rarely oblong-lanceolate, 3-5 veins, generally without secondary veins. ..... **b.** subsp. ***paniculatum***.

a) *Bupleurum rigidum* L. subsp. *rigidum*

Illustrations: H. Wolff in Engl., *Pflanzenr.* 43 (IV.228): 153, fig. 18 (1910); Fiori & Paol., *Iconogr. fl. ital.* (3 ed.): 274, fig. 2234 (1933); Valdés *et al.* (eds), *Fl. Andalucía Occid.* 2: 311 (1987); O. Bolòs & Vigo, *Fl. Països Catal.* 2: 443 (1990).

Basal leaves abruptly attenuate into ± long petiole, of up to 2/3 of total leaf length; leaf blade 0.7-6.5 cm broad, obovate, oblong-lanceolate or spatulate, 5-13 main veins, with numerous secondary veins.

World Distribution: Spain, SE Portugal (very rare), Ibiza?, S France, N Italy, Morocco & Algeria.

W Mediterranean Distribution: (see also map of distribution on p. 318)

Spain: A, Ab, B, Bu, Co, Cs, Cu, Ge, Gr, Gu, Hu, J, L, Le, Lo, M, Ma, Mu, Na, P, (PM [Ib]), S, Sa, Sg, So, T, Te, To, V, Va, Vi, Z, Za. *Portugal*: AAl. *Morocco*: 14. AS. *Algeria*: AS, H1, H2.

Note: *B. rigidum* subsp. *rigidum* is cited in the literature for Ibiza in the Balearic islands (see Beckett, 1988; and Romo, 1994).

Vernacular names: orella d'ase, orella de llebra, orella de llebre (Catalán), clujia basta, ontina, oreja de liebre (Spanish).

Representative specimens:

Spain: Albacete, Yeste, 15.viii.1985, C. Soriano (MA 462245); Alicante, S. del Cideret, 6.vii.1995, J. Soler & M. Signes (MA 561615); Barcelona, Mayans a Manresa, 9.vii.1988, R. Morales *et al.* (MA 458966); Burgos, Condado de Trevino, 3.ix.1983, P. Uribe & J. Alejandre (MA 417658); Castellón, Benicasim, 15.viii.1993, F. Cernoch & J. Schubertová (MA 563166); Córdoba, Almedinilla, 11.vi.1977, F. Casas & Muñoz Garmendia (MA 409595); Jaén, La Iruela, 17.viii.1976, González Rebollar *et al.* (MA 480217); Guadalajara, Albalate de Zorita, 6.viii.1978, E. Rico (MA 310467); La Rioja (Logroño), Torrecilla de Cameros, 8.viii.1978, F. Amich (SALA 13045); León, Vallecillo, 3.xii.1978, P. Montserrat (JACA 467278); Lérida, Cubells, vii.1983, J. Pedrol (MA 418513); Málaga, Sierra Bermeja, 18.vii.1975, J. Fernández Casas (MA 394828); Navarra, Torralba del Rio, 29.vii.1988, P.M. Uribe-Echebarria (MA 478801); Salamanca, S. Felices de los Gallegos, 21.ix.1977, F. Amich (SALA 16404); Toledo, Villatobas, cerros del arroyo Testillos, 1.vi.1982, S. Laorga (MAF 110576). **Portugal:** Alto Alentejo, Serra de S. Mamede, c. Picoto, 29.iv & 12.ix.1954, M. Béliz *et al.* (MA 325418). **Morocco: 14** - Metalsa, 27.v.1933, F. Sennen & H. Maurício (BM). **Algeria: OL** - Environs de Bossuet (Orán), 11.vii.1926, A. Faure (BM), & 16.vii.1933, A. Faure (MA 86542); Terny vers le Nador, 19.vii.1941, A. Faure (LISE).

b) *Bupleurum rigidum* L. subsp. ***paniculatum*** (Brot.) H. Wolff in Engl., *Pflanzenr.* 43 (IV.228): 154 (1910).

Basionym: *Bupleurum paniculatum* Brot., *Fl. lusit.* 1: 454-456 (1804).

Type: Neotype (selected here) – "Portugal: Beira Litoral, Conímbriga, mata da Abufarda (Alfarda, Abofada ou Bufarda).", 25.vii.1998, S.P. Neves & S.S. Neves 67 (COI! – neoisotypes in COI!, E!, K! & LISU!).

Type locality: "Hab. in collibus calcareis circa Conimbricam, et alibi in Beira et Extremadura."

Name origin: From the Latin '*paniculatus*', because the branching of the flowering stems resembles that of a panicle.

Illustrations: Valdés *et al.* (eds), *Fl. Andalucía Occid.* 2: 312 (1987).

Basal leaves sessile, not attenuate or gradually attenuate to the base, 0.5-1.2 cm broad, linear to linear-lanceolate, rarely oblong-lanceolate, 3-5 main veins, generally without secondary veins.

World Distribution: Portugal, W Spain & Morocco.

W Mediterranean Distribution: (see also map of distribution on p. 318)

Spain: Ca, Cc, (CR), (Gr), (J), Ma, Sa, Se, To, Za. *Portugal*: AAl, Ag, BAl, BL, E, R. *Morocco*: 1, 8, 9. CN, GA, MA. *Algeria*:?

Vernacular names: hierba dura, oreja de liebre (Spanish).

Representative specimens:

Spain: Cádiz, Sierra de Lijar, Algodonales, 28.vi.1980, A. Aparício (MA 504380); Cáceres, Sierra de Berzocana, 6.viii.1977, Pérez Chiscano (MA 208819); Málaga, Sierra del Torcal de Antequera, 26.vii.1961, Rivas Goday (MA 310446); Toledo, Navahermosa, 9.vii.1980, F. Amich (MA 310679); Zamora, entre Roda del Pan y Cubo del Vino, 1.ix.1983, S. Silvestre (MA 316101). **Portugal:** Alto Alentejo, Serra de S. Mamede, entre S. Salvador e Porto de Espada, 7.x.1970, M. Béliz & J.A. Guerra (MA 325424); Algarve, Loulé para S. Brás de Alportel, S. Romão, 15.v.1979, M. Béliz & J.A. Guerra (MA 325420); Baixo Alentejo, Ferreira do Alentejo, Beringel, 12.viii.1954, P. Silva & M. Silva (MA 325426); Beira Litoral, Eiras, 15.vi.1961, J. Paiva 30 (COI); Estremadura, Porto de Mós, 26.vii.1952, A. Fernandes *et al.* 4261 (COI); Ribatejo, Ourém to Torres Novas, c. Ponte do Furadouro, 31.vii.1994, S. Neves 6 (MA). **Morocco: 1** - Tanger, Pointe Cires, 25.viii.1970, Davis 50790 (E, BM, RNG); **9** -

NNE of Meknès, from Moulay Idriss to Nzaia-des-Beni-Ammar, 5.vi.1994, S.L Jury *et al.* 15010 (RNG); **MA** - Tinisiourine, 15 km WSW of Timhadit, 5.viii.1975, P. Crane / R.U. Biol. Exped. 68 (BM, RNG) [more correctly regarded as *B. rigidum* s.l.].

Typification notes:

A neotype was required for this taxon (subsp. *paniculatum*), because no original material exists of '*B. paniculatum*' from Brotero (Portuguese botanist, 1744-1828). It is believed that at the time of the French invasions of the Iberian Peninsula, Brotero, afraid that the French would steal his herbarium, preferred to destroy it. There are still some Brotero specimens in the herbaria of LISU and MO, but I did not find any specimen of '*B. paniculatum*' of his in LISU. I also checked in COI (Brotero was Professor of Botany in Coimbra). The curator of the Missouri Botanical Garden Herbarium, Dr James Solomon, kindly replied to my inquiry indicating that, despite looking through all their European material of this taxon, no material was found to have been collected or annotated by Brotero. The type specimens (neotype & isoneotypes) were collected in the type locality: Conímbriga (Portugal).

10.7 Species inadequately known and not typified

Although type material was revised for two of the following species, synonymy or recognition of the species status cannot be confidently established with the present knowledge of the morphological variation of *B. balansae* Boiss. & Reut., *B. oligactis* Boiss. and *B. montanum* Coss.

a) *Bupleurum antonii* Maire in *Bull. Soc. Sci. Nat. Maroc* 8: 133-134 (1928).

Type material: Syntypes – "Dr R. Maire - Iter Maroccanum Octavum 1924 - *Bupleurum antonii* n. sp. In Atlantis Majoris Ditione Mesfioua: in rupibus graniticis mont. Aouljdid, 2700m." (MPU!, P!).

Type locality: [Morocco] "Hab. in rupibus graniticis subalpinis Atlantis Majoris: in clivo septentrionali montis Aouljdid supra Ouinimsen et supra Tizi-n-Tichka, ad alt. 2600-3000 m".

Critical taxonomic notes:

This taxon is almost certainly a synonym of an earlier published name: all the syntypes seen can be identified as previously described taxa. Maire's description of this new species is hardly discriminatory; he stated that *B. antonii* is close to 5 other species (*B. montanum* Coss., *B. acutifolium* Boiss., *B. oligactis* Boiss, *B. balansae* Boiss. & Reut. and *B. mesatlaticum* Litard. & Maire), indicating one difference for each of these taxa, all of which were quantitative (number of veins and rays), several without gaps in the values ["radiis 6-12 (nec 3-5)" or "foliis basis 5-6 nervis (nec 7-11 nervis)"]. I am inclined to think that *B. antonii* is a synonym of *B. montanum* Coss. However, the type material includes at least two species, one seems to be *B. montanum* (not the most typical material), and the other is *B. benoistii* Maire – the latter is not in agreement with the description of *B. antonii*. However, part of the type material of *B. antonii* includes a few specimens which at present I cannot confidently identify, but that could correspond to *B. oligactis* or what has been regarded as *B. choulettii* (see below).

b) ***Bupleurum choulettii*** Pomel, *Nouv. mat. fl. atl.*: 140-141 (1874).

Type material: Syntypes – "Herbier Pomel"; "Université d'Alger. Herbier de l'Afrique du Nord. *Bupleurum choulettei.* Type!"; [other label] "339. *Bupleurum fruticescens* L. [...] 15 juillet 1858. Versant sud de la butte du télégraphe de Sétif a Constantine. Rec. par J. Choulette fils." (MPU!, P!).

Type locality: [Algeria] "Environs de Constantine et de Souk-Arhas".

Critical taxonomic notes:

This taxon has been regarded as a variety or a synonym of *B. oligactis*, and it could possibly be. However, part of the type material includes some specimens with crowded and very short leaves that I am not sure if they correspond to a different species or if only a phenotypic variant of *B. oligactis*. Study of additional collections from Algeria is necessary, which should also include *B. balansae* and *B. montanum*.

c) ***Bupleurum mesatlanticum*** Litard. & Maire in *Mém. Soc. Sci. Nat. Maroc* 26: 16-17 (1930).

Type locality: [Morocco] "Hab. in rupestribus basalticis Atlantis Medii ad fontes Suburis, prope fauces Kheneg Merzoul nuncupatas, ad alt. 1900 m".

Typification and taxonomic notes:

The authors in the protologue indicated that the type material was placed at AL, RAB and Litardière's herbarium – specimens of the latter can be found in G, LAU, MPU, P and RAB. No type material was found at MPU or P.

No singular discriminatory characters were indicated in the protologue; the description could possibly refer to *B. montanum* or *B. oligactis*.

11. Overall taxonomic and phylogenetic conclusions

The present taxonomic revision of *Bupleurum* L. in the W Mediterranean and Macaronesia, confirmed for many taxa the decisions of previous authors, in particular in Iberian/ European species. However, problems of species delimitation were found not only in several of the poorly known NW African endemics, but also in some of the much better studied taxa.

Most of the species/taxa under study needed typification, and, as this is essential for correct application of names, every effort was made to find and study the type material of all these taxa. Typification, with designation of type as holotype, lectotype or neotype, is presented here for all the delimited species and subspecies in the area; seventeen of the 29 species needed lecto or neotypifiying.

Species delimitation in *Bupleurum*

Iberian taxa

Morphological and molecular data (ITS sequences) strongly suggest that *B. fruticescens* L. and *B. spinosum* Gouan should be regarded as a single species (*B. fruticescens*). This was first proposed by Bolòs & Vigo (1971) who recognised these taxa at subspecies rank, but did not provide any explanation about this taxonomic decision.

All evidence, morphological and molecular, suggests that *B. bourgaei* Boiss. & Reut., a species so far regarded as an endemic to the SE mountains of the Iberian Peninsula, is indistinct from other populations of *B. ranunculoides* L., and should therefore be considered a synonym. Tutin (1968) had already suggested that this might be the case, but still regarded the taxon as a different species.

Bupleurum gerardii All. and *B. praealtum* L. are morphologically very closely related species, and distinction of the two taxa is often problematic. Molecular data also supports the species as close allies. Both morphological and molecular evidence indicates that a more detailed study of these taxa is necessary. This should include material from all the range of distribution of the species, as also of some other related species, such as *B. commutatum* Boiss. & Balansa and *B. trichopodum* Boiss. & Spruner.

Although there is no clear morphological distinction between the material of the two populations of *B. acutifolium* Boiss. (Portuguese & Spanish), the ITS sequences obtained are far too different to be regarded as those of a single species. Further research is necessary to ascertain if these two geographically distant populations correspond to different species.

Balearic Islands

Bupleurum barceloi has been considered in recent works a subspecies of *B. dianthifolium* Guss. (Bolòs & Vigo, 1971, 1990), an endemic to the island of Marettimo, S of Sicily. However, these two species are clearly distinct, and morphologically *B. barceloi* is far closer to *B. acutifolium*, which is further supported by the ITS molecular data.

NW African taxa

Bupleurum balansae Boiss. & Reut. has often been regarded as very closely related or a synonym of *B. fruticescens* L.; but these taxa are clearly distinct, both morphologically and regarding the ITS sequences. However, I am not satisfied with my definition of *B. balansae*, as molecular data suggests that I am probably using inadequate morphological characters to recognise the taxon, possibly including more than one species under this name.

Bupleurum atlanticum Murb. and *B. oligactis* Boiss. have been regarded as distinct species, however the characters that are said to distinguish them vary considerably, even within a single specimen. I therefore regard *B. atlanticum*, a later name, as a synonym of *B. oligactis*.

Morphological distinction between *B. oligactis* and *B. montanum* is not always clear, with some material appearing to be intermediate between the two species, possibly meaning that hybridization is occurring (chromosome number records also show considerable aneuploidy in these populations – see Cauwet, 1979b). ITS sequences of these taxa differ in about 6 bp, which indicates that they are closely related species.

Therefore, further research is necessary at the population level in some of the NW African endemics, in particular *B. balansae*, *B. montanum* and *B. oligactis*, to more fully understand the range of morphological variation within these taxa, and the influence of the environment. Then, it would be possible to more confidently

establish to which material the names *B. choulettii* Pomel, *B. mauritanicum* Batt., and *B. mesatlanticum* Litard. & Maire should be applied, and if any of these correspond to different species. It would also be important to include material not only from Morocco, but also from Algeria and Tunisia from where new collections and field survey are urgently needed.

Macaronesian taxa

I have not found any evidence that would clearly indicate that *B. handiense* (Bolle) G.Kunkel, a presumed endemic in the Canary Islands (Fuerteventura and Lanzarote), is a distinct species from *B. canescens* Schousb. (endemic to S Morocco). So, the original varietal rank attributed to this taxon appears to be more appropriate (leaves appear to be broader in '*B. handiense*').

The morphological and anatomical differences between the populations of *B. salicifolium* R.Br. ex Buch (Madeira & Canary Islands) are small and do not merit subspecific rank, as it has been considered in recent works (Cauwet & Sunding, 1981; Hansen & Sunding, 1993).

Phylogenetic relationships in *Bupleurum*

Morphology has provided only a few distinctive and reliable characters in the investigation of relationships in the genus (see chapter 6). Also, morphological characters can be misleading about relationships – convergence and parallelism are known to occur in many plant groups. Therefore, it is indispensable to obtain evidence from other sources, in particular genetic data, in order to ascertain relationships or verify those that are suggested by morphology.

The present molecular work is the first comprehensive phylogenetic investigation in the genus. The work of Choi *et al.* (1996) included only 4 Asian species, and several other studies (see section 3.7) including a few species of *Bupleurum* were essentially concerned with the suprageneric classification in the *Umbelliferae* (subfamily *Apioideae*).

Phylogenetic analysis of sequences of the ITS region of nuclear ribosomal DNA repeat in *Bupleurum* clearly indicates that the genus is divided into two main groups. This division is supported by analyses of ITS1 and ITS2 (rapid evolving genes) and also the 5.8S subunit (a conserved coding region). A representative sample of the species in the genus was sequenced, and so these results have

implications in the classification that go beyond the species under study. The present results do not support the currently accepted classification of the genus in 5 sections (*Bupleurum*, *Diaphyllum*, *Reticulata*, *Isophyllum* and *Coriacea*), as neither support the highly questionable classification proposed by Cauwet (1976) – see sections 2.3 (p. 17) and 9.4 (p. 180-181).

Therefore, two new subgenera are proposed:

Subgenus *Penninervia* S.S.Neves (*subgenus nov.*), is a small basal group including all the species with pinnate-reticulate leaves, and also, surprisingly, *B. rigidum* L., a species morphologically quite distinct from the others included in this group. These species are found only in the Mediterranean region.

Subgenus *Bupleurum* as defined here includes the vast majority of the species of the genus, all of which have ± parallel-veined leaves.

The main clades (groups) within these subgenera are unresolved or weakly supported by the ITS analyses. Therefore, as neither morphological or molecular data are conclusive, the groups within each subgenus are informally treated. However, two groups within subgenus *Bupleurum* are strongly supported by both molecular and morphological characters: '*Perfoliata*' and '*Trachycarpa*' (the first presently regarded as Sect. *Bupleurum*, and the second as a subsection of Sect. *Isophyllum* – see chapter 2). A third group within subgenus *Bupleurum*, which I designated as the 'NW African origin' group, is also strongly supported by the ITS analyses. This new taxonomic group includes all endemics in NW Africa, and also all non-African species that are morphologically close to some of the NW African endemics. Also, the low nucleotide variation of ITS in this group indicates that these taxa may have recently radiated. There is no singular morphological character joining the taxa of the 'NW African origin' group (some of the species are morphologically quite distinct – e.g. *B. album* Maire), but a conjunction of morphological characters, together with the 'close' geographical distribution, appear to distinguish the group from other taxa within the genus (see also the phenetic ordination analysis in section 6.3.3). However, further molecular investigation, including genes other than ITS, is needed before designating appropriate taxonomic ranks for any of these groups.

The results of phylogenetic analyses indicate that the genus has probably originated in the W Mediterranean, because the most basal ('primitive') group,

subgenus *Penninervia*, has essentially a W Mediterranean distribution. Also, *Bupleurum* shows the highest diversity in morphological patterns in this region, which includes all its woody species (woodiness is probably an ancestral state).

The Macaronesian endemic *B. salicifolium* was found to be a neoendemic as the ITS sequence divergence to its continental relatives is quite small. Another important finding is that ITS data indicates that *B. mundii* Cham. & Schltdl., a vicariant species in S Africa, is a neoendemic closely related to *B. falcatum* L. (an Eurasian species). Therefore, *B. mundii* is not a remnant of a more widespread distribution of *Bupleurum* in Africa – the phylogenetic placing of this species has been questioned before when considering the possible links of the genus to other basal woody *Apioideae* genera, all mainly from C & S Africa.

Final phylogenetic analyses including sequences from 4 other basal *Apioideae* genera (*Anginon* Raf., *Heteromorpha* Cham. & Schltdl., *Physospermum* Cusson ex Juss. and *Pleurospermum* Hoffm.) confirms *Bupleurum* as a monophyletic group. However, none of these genera appeared particularly close to *Bupleurum* in the ITS analyses; and neither there are clear morphological or anatomical characters associating the genus to these or other basal genera in *Apioideae*. Several molecular studies that have been carried out in the *Umbelliferae* indicate that further molecular data need to be obtained to resolve the relationships between *Bupleurum* and the other basal genera in subfamily *Apioideae*.

Potentially useful sources of taxonomic data in *Bupleurum*

Morphology will always be one of the main sources of taxonomic data, in particular to delimit species and populations. However, it is essential that the herbarium or other plant collections studied represent the variability of the taxa. The NW African collections are at the present insufficient, and further morphological study on the problematic taxa in the area is necessary. So, it is fundamental to carry out further **field surveys**, with more detailed records on the general morphology of specimens (e.g. habit and height), but also on their habitat and ecology, and the relative frequency of the plants in the area. Part of the variation I observed in some of the species appears to be environmentally related, but usually very little is said about

the habitats. Collection of fruit material would also be necessary, not only for conservation purposes, but also for study of living plants under cultivation.

Anatomy has provided some interesting characters in *Bupleurum*, in particular in fruits. However, additional anatomical work including a representative sample of the species, is necessary to determine the taxonomic relevance of some of these fruit characters. Also, stem and leaf anatomy can potentially provide useful characters in the investigation of relationships among the taxa.

SEM studies do not seem to be good sources of taxonomic data in the genus, because of the lack of superficial structures on the epidermis of *Bupleurum* (no hairs or glands), the fact that stomata characteristics seem unreliable, and that pollen shows little variation.

Karyology in particular **chromosome morphology**, is a domain of research that can provide very valuable data for the study of populations and the relationships between taxa. However, collections of fruits and living plants need to be increased, as at the moment they are scarce, and generally do not include the problematic species.

Molecular investigation in the genus is at the very beginning, but is potentially the most important source of data to determine relationships. It is necessary to increase the number of species sequenced for ITS, especially to include E Mediterranean and Asian taxa. It is essential to extensively sequence other genes to confirm and to improve the resolution of the present phylogenetic analyses. Methods other than DNA sequencing, such as RFLPs, AFLPs, microsatellites, and allozyme studies (see section 9.1) can also provide useful data a different levels on the classification of *Bupleurum*.

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Appendices

Appendix I

Herbaria abbreviations and addresses

Standard abbreviations and general information on herbaria are according to *Index Herbariorum* (8 ed.), P. K. Holmgren *et al.* (eds) (1990). Name of curators or keepers, when indicated, have been recently confirmed or updated for several herbaria.

AL - Herbarium, Laboratoire de Botanique de la Faculté des Sciences, Université d'Alger, Algiers, Algeria.

AVE - Herbário, Departamento de Biologia, Universidade de Aveiro, 3800 Aveiro, Portugal. *Curator*: Rosa Pinho.

BC - Herbario, Institut Botànic de Barcelona, Av. dels Muntanyans s.n., Parc de Montjuìc, 08004 Barcelona, Spain. *Curators*: N. Escué and À.M. Romo.

BCF - Herbario, Laboratori de Botànic, Facultat de Farmàcia, Universidat de Barcelona., Av. Diagonal s.n., 08028 Barcelona, Spain. *Curators*: J. Molero-Briones and M.A Ribera-Siguan.

BM - Herbarium, Botany Department, The Natural History Museum, Cromwell Road, London SW7 5BD, England, UK. *Keeper*: Stephen Blackmore.

C - Herbarium, Botanical Museum, University of Copenhagen, Gothersgade 130, DK-1123, Copenhagen, Denmark. *Curators*: Bertel Hansen and Olof Ryding.

COI - Herbário, Departamento de Botânica, Universidade de Coimbra, 3049 Coimbra, Portugal. *Curator*: Fátima Sales [Maria F.M. Sales Machado].

E - Herbarium, Royal Botanic Garden, Inverleith Row, Edinburgh EH3 5LR, Scotland, UK. *Curator*: David F. Chamberlain.

FI - Herbarium Universitatis Florentinae, Museo Botanico, Via G. La Pira 4, I-50121 Firenze, Italy. *Curators*: Piero Cuccuini and Chiara Nepi.

G - Herbarium, Conservatoire et Jardin Botaniques de la Ville de Genève. Case postale 60, CH-1292 Chambésy/Genève, France. *Curator*: F. Jacquemoud [previously C. Charpin].

GDA - Herbario, Departamento de Biología Vegetal (Cátedra de Botánica), Facultad de Farmácia, Universidad de Granada, 18071 Granada, Spain. *Curator*: M.C. Quesada Ochoa.

K - Herbarium, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3AB, England, UK. *Keeper*: S. J. Owens.

JACA - Herbario, Unidad de Geobotánica, Instituto Pirenaico de Ecología, CSIC. Jaca (Huesca). *Curator*: J.D. Gómez.

LAU - Herbarium, Musée et Jardins Botanique Cantonaux, Avenue de Cour 14 bis, CH-1007 Lausanne, Switzerland. *Curator*: Jean-Louis Moret.

LD - Herbarium, Botanical Museum, Ö. Vallgatan 18, S-223 61 Lund, Sweden. *Curator*: Per Lassen.

LINN - Herbarium, Linnean Society of London, Burlington House, Piccadilly, London W1W OLQ, England, UK. *Correspondent*: Gina Douglas.

LISE - Herbário, Fitossistemática e Geobotánica, Estação Agronómica Nacional. *Curator*: M. Isabel S. Costa.

LISI - Herbário, Departamento de Botânica, Instituto Superior de Agronomia, 1399 Lisboa, Portugal. *Correspondent*: João Amaral Franco.

LISU - Herbário, Instituto Botânico, Faculdade de Ciências, Universidade de Lisboa, Rua Escola Politécnica, 1294 Lisboa Codex, Portugal. *Curator*: Alexandra S. C. Escudeiro.

MA - Herbario, Real Jardín Botánico de Madrid, CSIC, Plaza de Murillo 2, 28014 Madrid, Spain. *Curator*: Mauricio Velayos.

MADJ - Herbário, Jardim Botânico do Funchal, Secção de Fitotaxia/Fitoteca, 9000 Funchal, Madeira, Portugal. *Curator*: Rui A.G. Santos.

MAF - Herbario, Cátedra de Botánica, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain. *Curator*: J. Pizarro Domínguez.

MGC - Herbario, Departamento de Biología Vegetal, Universidad de Málaga. *Curator*: J.M. Nieto-Caldera.

MO - Herbarium, Missouri Botanical Garden, P.O. Box 299, Saint Louis, Missouri 63166-0299, USA. *Curator*: James C. Solomon.

MPU - Herbier, Institut de Botanique, 163 rue Auguste Broussonnet, F-34000 Montpellier, France. *Curator*: Peter S. Schäfer.

P - Herbier, Laboratoire de Phanérogamie, Muséum National d'Histoire Naturelle, 16 rue Buffon, F-75005 Paris, France. *Curator*: J. Jolinon.

RAB - Herbarium, Institut Scientifique Département de Botanique et d'Ecologie Végétale, Av. Ibn Batteta, B.P. 703, Rabat, Agdal, Morocco.

RNG - Herbarium, Plant Science Laboratories, University of Reading, P.O. Box 221. Whiteknights, Reading, Berkshire RG6 2AS, England, UK. *Curator*: Stephen L. Jury.

S - Herbarium, Botany Departments, Swedish Museum of Natural History, P.O. Box 50007, S-104 05 Stockholm, Sweden. *Correspondent*: Bertil R. Nordenstam.

SALA - Herbario, Facultad de Biología (Botánica), Universidad de Salamanca, 37008 Salamanca. *Curator*: J. Hernández García.

SALAF - Herbario, Biologia Vegetal (Botánica, Farmacia), Universidad de Salamanca, Av. Campo Charro s.n., 37007 Salamanca, Spain. *Curator*: Cipriano J. Valle.

SEV - Herbario, Departamento de Botánica, Facultad de Biología, Universidad de Sevilla. Apartado de Corroes 1095, 41080 Sevilla, Spain. *Curator*: C. Romero Zarco.

TFC - Herbario, Departamento de Botánica, Universidad de La Laguna, Apartado 38271, La Laguna, Tenerife, Canary Islands, Spain. *Curator*: J.R. Acebes-Ginovés.

TFMC - Herbario, Departamento de Botánica, Museo de Ciencias Naturales, Apartado Correos 853, 38080 Santa Cruz de Tenerife, Canary Islands, Spain. *Curator*: Lázaro M. Sánchez-Pinto.

TLON - Herbier Musée d'Histoire Naturelle de la Ville de Toulon. 113 Boulevard Marechal Leclerc. F-38000 Toulon, France.

UPS - Botanical Museum (Fytoteket), Uppsala University, P.O. Box 541, S-751 21 Uppsala, Sweden. *Correspondent*: Roland Moberg.

Appendix II

Accession numbers cited

The following table gives detailed information for the accession numbers cited in the text. If known, country of origin (in italic) is (generally) referred first, followed by province or region, and then locality; name of collector is underlined. See Appendix I for explanation of herbarium abbreviations (in brackets). Some herbaria (e.g. in Spain) have their own numbering of specimens; in these cases such number is given after herbarium abbreviation. Hedge & Lamond (1970) was consulted to verify some names of collectors or collection dates of specimens from the Edinburgh herbarium. All herbarium specimens were seen by myself.

Taxa	Acc. No	Origin of plant material
Anginon difforme (L.) B.L.Burtt	**191**	*South Africa*: Draakensteansbergen, 1000-2000 m, x.[1826-37?], J.F. Drège (E).
	192	*South Africa*: Port Grahamston, marshy ground near river, [date?], G.F. Scott Elliot (E).
	193	*South Africa*: Calvinia, Hantam Mountains, Farm Vanrhynshoek, stream bank, 1600 m, 21.viii.1990, All Batten AB 1018 (E).
A. paniculatum (Thunb.) B.L.Burtt	**313**	*South Africa*: "Central ridge" above Clanwilliam, dam 5 km from Clanwilliam, in fine sand, 18.ii.1985, H.C. Taylor 11271 (E).
Bupleurum acutifolium Boiss.	**228**	*Portugal*: Baixo Alentejo, Serra do Cercal, hills bordering road from Vila Nova de Milfontes to Cercal, 37°48' N - 8°40' W, 16.ix.1996, S. Neves 24 (COI, E).
	240	*Portugal*: Baixo Alentejo, Serra de S. Domingos (*Eucalyptus globulus* culture), near S. Luis, 37°43' N - 8°40' W, 200-300 m, 08.viii.1997, S. Neves 27 (COI, E).
	262	*Spain*: Málaga, Sierra Bermeja, margin of road (in construction) from Peñas Blancas to 'Refugio', in direction to Los Reales, c. 1030 m, 05.ix.1997, S. Neves 64 (COI, E).
	263	*Spain*: Málaga, Sierra Bermeja, on road from Estepona to Puerto de Peñas Blancas, c. 800 m, 05.ix.1997, S. Neves 65 (E).
	305	*Spain*: Málaga, Tolóx, Sierra Parda, Majada Redonda, 770 m, 06.v.1994, A. Pérez-Latorre *et al.* (MGC 37708).
B. album Maire	**269**	*Morocco*: Anti-Atlas, 4 km from Igherm, road to Taliouine, 10.vi.1974, Reading University / BM Expedition 532 (RNG).
B. angulosum L.	**174**	Pyrenees. Mericarps collected from cultivated material; received from the Botanischer Garten, University of Bern, Switzerland (Index Seminum 1994).

Taxa	Acc. No	Origin of plant material
Bupleurum angulosum L.	**224**	Pyrenees. Material collected from living plant at the Royal Botanic Garden Edinburgh (Rock Garden - R25) – RBGE Acc. No 19861043. Voucher specimen: S. Neves s.n. (E).
B. balansae Boiss. & Reut.	**268**	*Morocco*: Oujda (area 19), Al'Youn, Machra, Homadi, Oued Senara, 34°44' N - 2°45' W, 450 m, bushes, 09.vi.1993, J. Molero *et al.* JMM-3198/5 (RNG).
	302	*Morocco*: S Tetouan (area 2), c. 35 km S of Tetouan, along main road to Chechaouen, near Souk-el-Arba-des-Beni-Hassan, 35°33' N - 5°22' W, 320 m (French Lambert North Morocco Grid 503550), 18.vi.1987, S.L. Jury *et al.* 8338 (RNG).
B. baldense Turra	**276**	*Spain*: Guadalajara, Checa, river Cabrillas, road from Checa to Orea, km 3.5, 1450 m, 21.vi.1995, M.A. Carrasco *et al.* (MA 558704).
B. barcelol Coss. ex Willk.	**295**	Balearic Islands (*Spain*), Mallorca, Sóller, "Barranco" (cliff) 25.vii.1989, J. Orell Casasnovas (MA 474781).
B. benoistii Litard. & Maire	**285**	*Morocco*: High Atlas, S from Marrakech, N end of Jbel Oikaïmeden near azib in ski resort, 31°12' N - 7°52' W, 2700 m, open heavily grazed area, 29.vii.1997, S.L. Jury *et al.* 18375 (E).
	300	*Morocco*: prov. of Ksar el Souk, N High Atlas, Tizi n'Tirrecht, N of Ari n'Ayachi, near Midelt, c. 10,000', growing in soil in a rocky gully, 21.vii.1966, R.M. & A.M. Harley 766 (BM).
	309	*Morocco*: 72 km S from Marrakech, Oukaïmeden, 31°13' N - 7°52' W (French Lambert North Morocco Grid 264072), 2700 m, SE facing rocky slopes, 03.vii.1987, S.L. Jury *et al.* 8858 (RNG).
B. canescens Schousb.	**301**	*Morocco*: Immouzer Valley, N of Agadir, dense maquis, cliffs and steep rocky slopes, 28.iii.1972, D. Bramwell *et al.* 265 (RNG).
B. canescens Schousb. var. ***handiense*** Bolle [= *B. handiense* (Bolle) G.Kunkel]	**28**	Canary Islands (*Spain*). Mericarps collected in the wild, received from the Jardín Botánico "Viera y Clavijo" (Index Seminum 1993). Voucher specimen: S. Neves s.n. (COI).
	207	Canary Islands (*Spain*), Lanzarote, Peñas de Chache, 600 m; rare shrub on cliffs above Famara, 15.v.1969, D. Bramwell 1631 (E).
B. dumosum Coss. & Balansa	**293**	*Morocco*: c. 9.5 km NNE of Asni, 5 km SSW of Tahanaoute, Gorge de Moulay Brahim, 31°19' N - 7°58' W, 1050 m, rock crevices, 15.iii.1994, S.L. Jury *et al.* 14157 (RNG).
B. falcatum L.	**118**	Mericarps collected from cultivated material; received from the Jardin Botanique, Section Pharmacie, Angers, France (Index Seminum 1994).

Taxa	Acc. No	Origin of plant material
Bupleurum falcatum L.	**282**	*Spain*: Alava, Lagrán, Sierra de Cantabria, rocky crests of Recilla, 1330-1340 m, limestone, 15.viii.1992, J.A. Alejandre 733/92 (MA 534085).
B. foliosum Salzm.	**275**	*Spain*: Cádiz, Los Barrios, Arroyo del Prior, 400 m, 31.v.1981, J. Arroyo & J.M. Gil (SEV 69040).
	278	*Spain*: Málaga, Estepona, road up to Peños Blancos, 500 m, serpentines, 14.vii.1988, B. Cabezudo *et al.* (MGC 23807).
B. fruticescens L. subsp. ***fruticescens***	**238**	*Spain*: Huesca, between Baldellou and Camporrels, 550-570 m, 30.ix.1987, G. Montserrat (MA 515853).
	253	*Spain*: Murcia, Sierra de Espuña, margins of road up to "Centro de Interpretación", c. 770 m, calcareous soil, pine forest, 24.viii.1997, S. Neves 52 (COI, E).
B. fruticescens L. subsp. ***spinosum*** (Gouan) O.Bolòs & Vigo	**34**	*Spain*: Cádiz, Grazalema, Gomez-Campo s.n. (seed bank Madrid). Mericarps collected from cultivated material at the Botanischer Garten und Botanischer Museum, Berlin-Dahlem; collected prior 1992 [mostly 1991].
	155	Mericarps collected from cultivated material; received from the Jardin Botanique de Lausanne, Switzerland (Index Seminum 1993).
	169	Mericarps collected from cultivated material (outdoors); received from the Botanischer Garten der Christian-Albrechts Universität, Kiel, Germany (Index Seminum 1993/94).
	249	*Spain*: Granada, Sierra Nevada, Monachil valley, high mountain, c. 1250 m, 16.viii.1997, S. Neves 42 (COI, E).
	259	*Spain*: Cádiz, Zahara to Grazalema, margins of road CA-531, c. 1100 m, calcareous rocky soil, open vegetation, 22.viii.1997, S. Neves 47 (COI, E).
	280	*Morocco*: High Atlas, S from Marrakech, ski resort of Oukaïmeden, 31°13' N - 7°51' W, 2620 m, rock crevice above lake, 27.vii.1997, S.L. Jury *et al.* 18297 (E).
	311	*Morocco*: W Rif (area 2), Chefchaouene, Djebel Bouhalla to Djebel Lakraa, 35°06' N - 5°09' W, 1250 m, basic soil, 23.vii.1995, M.A. Mateos *et al.* 6988/95 (RNG).
B. fruticosum L.	**5**	Mericarps collected from cultivated material; received from the Jardin Botanique Université Louis Pasteur, Strasbourg, France (Index Seminum 1993).
	7	Mericarps collected in 1991 from cultivated material; received from the Jardin Botanique de la Ville de Nice, France.

Taxa	Acc. No	Origin of plant material
Bupleurum fruticosum L.	**15**	*Portugal*: Estremadura, Serra da Arrábida, xi.1992. Mericarps obtained from the Jardim Botânico da Universidade de Lisboa (Lisbon); collected in the wild.
	61	*Spain*: Jaén, Arguillos, road to Úbeda, 620 m. Mericarps obtained from the Real Jardín Botánico de Madrid, Spain; collected in the wild (Index Seminum 1993).
	119	Mericarps collected from cultivated material; received from the Jardin Botanique, Section Pharmacie, Angers, France (Index Seminum 1994).
	243	*Portugal*: Estremadura, Serra da Arrábida, slopes bordering road from Aldeia de Irmaõs to Casais da Serra, 38°30' N - 9°01' W, c. 100 m, 10.viii.1997, S. Neves 33 (E).
	248	*Spain*: Málaga, Sierra Bermeja, road Ronda to Puerto de Alijar, margin of stream, c. 525 m, 15.viii.1997, S. Neves 41 (E).
B. gerardii All.	**17a**	Mericarps collected from cultivated material (indoors); received from the Jardin Botanique National de Belgique, Meise, Belgium (Index Seminum 1992). Voucher specimen: S. Neves s.n. (E).
	44	*Portugal*: Beira Litoral, Coimbra, Casa do Sal, 15.vii.1993. Mericarps obtained from the Jardim Botânico, Universidade de Coimbra, Portugal; collected in the wild.
	67	*Portugal*: Beira Litoral, Fátima, Valinhos, in the area of 'Via Sacra', 39°37' N - 8°40' W, 380 m, calcareous soil, open Mediterranean vegetation, 26.vi.1994, S. Neves 1 (COI, E, MA).
	69	*Idem* (mericarps collected 16.vii.1994).
	306	*Spain*: Madrid, 'embalse' [dam] of Santillana, Cerro Casal, 10.vii.1981, G. Navarro *et al.* (MA 310732).
	307	*Spain*: Granada, Lobras, Barranco de los Lagartos, rocky slope, 7.v.1980, J. Molero Mesa (MA 214590).
B. gibraltarium Lam.	**183**	Mericarps collected from cultivated material; received from the Real Jardín Botánico de Madrid, Spain (Index Seminum 1994).
	245	*Spain*: Sevilla, near Coripe, road to Algodonales, calcareous slopes bordering river Guadalporcún, 500-550 m, 15.viii.1997, S. Neves 35 (E).
	252	*Spain*: Murcia, Sierra de Espuña, margins of road to 'Centro de Interpretación', calcareous soil, pine mixed forest, 24.viii.1997, 24.viii.1997, S. Neves 51 (COI, E).

Taxa	Acc. No	Origin of plant material
Bupleurum lancifolium Hornem.	**45**	*Portugal*: Beira Litoral, Coimbra, Póvoa das Pegas, 29.vii.1993. Mericarps obtained from the Jardim Botânico, Universidade de Coimbra, Portugal; collected in the wild.
	73	*Palestine*: Tiberias, 1877, J. Ball (E).
	74	*Turkey*: Urfa province, Urfa to Akçakale, 450 m, Davis & Hedge D2812 (E).
	75	*Lebanon*: Beirut, 1871 [no name of collector] (E).
	76	*Egypt*: lower Egypt, Ramli, Letourneux 63 (E).
	287	*Morocco*: Tanger (area 1), SW of Chefchaouen, 2.3 km up road to Mokrissèt from Pont du Loukos, 35°00' N - 6°77' W, 360 m, in gully under olive trees, 21.iv.1995, S.L. Jury *et al.* 16552 (RNG).
B. lateriflorum Coss. ex H.Wolff	**279**	*Morocco*: High Atlas, S from Marrakech, 4 km below Oukaïmeden, on road to Vallée de l'Ourika, 31°14' N - 7°50' W, 2380 m, sandstone cliffs, 28.vii.1997, S.L. Jury *et al.* 18323 (E).
	303	*Morocco*: High Atlas, Tizi-n-Test Pass, 30°52' N - 8°23' W (French Lambert South Moroccan Grid 216434), 2065 m, shale bank, 02.x.1991, M. Ait Lafkih *et al.* 4939 (E).
B. longifolium L.	**31**	*Germany*: Baden-Württemberg, Schwäbische, 900 m, Raus s.n. Mericarps collected from cultivated material; received from the Botanischer Garten und Botanischer Museum, Berlin-Dahlem; collected prior 1992 [mostly 1991].
	165	Mericarps collected from cultivated material; received from the Botanischer Garten/ Jardin Botanique, Fribourg, Switzerland (Index Seminum 1994).
	310	*Germany*: Baviera, Oberpfalz, Kreis Regensburg, 0.3 km WSW from Pentling, N of road from Pentling to Weichsmühl, margins of river Donau, c. 400 m, on limestone, locally common, 17.vii.1993, H. Förther 7503 (MAF 149194).
B. montanum Coss.	**264**	*Morocco*: Chefchaouen (area 7), between Ketama and Bab-Berret, 34°58' N - 4°43' W, 2000 m, *Cedrus atlantica* forest, 03.xi.1993, P. García Murillo *et al.* ST 251/93 (SEV).
	292	*Morocco*: High Atlas, just above El-Ksiba, along road to Imilchil, 32°31' N - 6°01' W, 1000 m, on limestone, woodland, 5.vii.1997, S.L. Jury 17456a (E).
	304	*Morocco*: Checfchaouen (area 2), Djebel Tassaot, 35°17' N - 5°07' W, 1550 m, on limestone, 'pinsapar' [*Abies pinsapo* woodland?], 22.vii.1995, M.A. Mateos *et al.* 6914/95 (SEV).

Taxa	Acc. No	Origin of plant material
Bupleurum mundii Cham. & Schltdl.	**283**	*South Africa*: Natal. Material collected from cultivated plants at the Royal Botanic Garden Edinburgh (Q13) – RBGE Acc. No 19972669; mericarps originally collected by the Lesotho/ Edinburgh/ Gothenburg Expedition 1997 (No 316). Voucher specimen: S. Neves s.n. (E).
B. odontites L.	**291**	*Tunisia*: Kroumirie, c. 14 km N from Jendouba (Souk el Arba) to Ain Draham, S of Tabarka, 210 m, fallow clay fields in rich herbage, 11.v.1975, Davis & Lamond D57628 (RNG).
B. oligactis Boiss. [*B. atlanticum* Murb.]	**92**	*Morocco*: High Atlas, 111 km N from Errachidia (Ksar-es-Souk) along P21 road to Midelt, a few kms S of Tizi-m-Tairhemt, 32°33' N - 4°29' W (French Lambert North Morocco Zone 586218), 1875 m, open dry *Stipa tenuissima* grassland, 12.vii.1987, S.L. Jury *et al.* 9240 (MA 391152).
	265	*Idem*; S.L. Jury *et al.* 9240 (SEV 127166).
	281	*Morocco*: Middle Atlas, road from El-Ksiba to Imilchil, c. 9 km N from Tizi-n-Islay, 32°23' N - 5°45' W, 1440 m, E-facing bank with clearings and fields amongst scrub, 05.vii.1997, S.L. Jury 17516 (E).
	298	*Morocco*: High Atlas, about 3 km above Imilchil, on road to lake Tizlite, along El-Ksiba to Imilchil road, 32°11' N - 5°39' W, 2160 m, open *Stipa* community with young planted *Cupressus*, 7.vii.1997, S.L. Jury 17603 (E).
B. plantagineum Desf.	**272**	*Algeria*: K2, Cap Carbon, near Bejaïa (Bougie), 250 m, rocky N limestone slope, maquis, 29.v.1991, Davis 52959 (RNG).
B. praealtum L.	**3**	Mericarps collected from cultivated material (outdoors); received from the Botanischer Garten St. Gallen, Switzerland (Index Seminum 1992).
	112	*Idem* (Index Seminum 1993).
	267	*Spain*: Lérida, Valle de Boí, Barruera, 2 km from the village in direction to Pont de Suert, 1200 m, calcareous slopes, 23.viii.1987, C. Aedo *et al.* 286-87 ML (MA 449973).
	288	*Spain*: Huesca, San Juan de Plan, near 'ermita' [hermitage] of San Mamés, 1350 m, siliceous soil, 01.viii.1981, P. Montserrat *et al.* (JACA 191581).
	289	*Spain*: Teruel, Loscos, Piedrahita, river Noguera, 1000 m, 13.viii.1995, C. Fabregat & Lopez Udias (JACA 683295).
	308	*Spain*: Salamanca, Montemayor del Rio, 2.viii.1983, J.L. Fernández Alonso & A. Guillen (MA 518939).

Taxa	Acc. No	Origin of plant material
Bupleurum ranunculoides L.	**43**	Mericarps collected from cultivated material; received from the Botanischer Garten Tübingen, Germany (Index Seminum 1992).
	181	Mericarps collected from cultivated material; received from the Botanischer Garten der Universität Postdam, Germany (Index Seminum 1993/94). Voucher specimen: S. Neves s.n. (E).
	296	*Spain*: Burgos, Rebolledo de la Torre, Peña Castro, 1340 m, calcareous crests, 24.vi.1990, J.A. Alejandre 1078/90 (MA 493699).
	297	*Spain*: Jaén, Pontones, Sierra de Banderillas, 1600 m, calcareous fissures, 10.viii.1982, C. Soriano (MA 462383) – [as *B. bourgaei* Boiss. & Reut.].
B. rigidum L. subsp. ***rigidum***	**190**	*Spain*: Barcelona, massif of Tibidabo, Barranco de S. Genis, 14.ix.1912, F. Sennen 1391 (E).
	254	*Spain*: Murcia, Sierra de Espuña, picnic area near "Centro de Interpretación", c. 740 m, pine forest, 24.viii.1997, S. Neves 53 (COI, E).
	261	*Spain*: Málaga, Sierra Bermeja, margin of road from Estepona to Puerto de Peñas Blancas, c. 700 m, 05.ix.1997, S. Neves 63 (COI, E).
B. rigidum L. subsp. ***paniculatum*** (Brot.) H.Wolff	**70**	*Portugal*: Beira Litoral, Coimbra, Quinta da Sapata, old national road Coimbra-Lisboa, 2 km from Santa Clara, 40°10' N - 8°26' W, 180 m, calcareous soil, open Mediterranean vegetation, 27.vi.1994, F. Sales & S. Neves 3[a-c] (E).
	86	*Portugal*: Beira Litoral, S. Sebastião, slopes beside new road Ourém – Fátima, 39°38' N - 8°36' W, 160-300 m, calcareous soil, open Mediterranean vegetation, 31.vii.1994 (mericarps collected 12.ix.1994), S. Neves 8[a-b] (E).
	87	*Portugal*: Beira Litoral, Cabo Mondego, viewpoint near the old light-house, 40°10' N - 8°54' W, 45-50 m, calcareous soil, open Mediterranean vegetation, 02.viii.1994 (mericarps collected 16.ix.1994), S. Neves 9-10 (COI).
	89	*Portugal*: Beira Litoral, Fátima, Quinta do Poço Soudo, 260 m, calcareous soil, Mediterranean oak wood, 22.ix.1994, S. Neves 25 (COI).
	234	The same as Acc. No 86 (mericarps collected 12.x.1996).
	244	*Portugal*: Estremadura, Serra da Arrábida, Vale da Rasca, margin of road in direction to the 'Secil' cement factory, 38°30' N - 8°57' W, c. 80 m, calcareous soil, open Mediterranean vegetation, 10.viii.1997, S. Neves 31 (E).

Taxa	Acc. No	Origin of plant material
Bupleurum rotundifolium L.	**4**	Mericarps collected from cultivated material (outdoors); received from the Botanischer Garten St. Gallen, Switzerland (Index Seminum 1992). Voucher specimen: S. Neves s.n. (E).
	13	Mericarps collected from cultivated material; received from the Botanischer Garten München-Nymphenburg, Germany (Index Seminum 1992).
	171	Mericarps collected from cultivated material; received from the Botanischer Garten der Universität Erlanger, Germany (Index Seminum 1994 – as " *B. griffithii* ").
B. salicifolium R.Br. ex Buch	**29**	Canary Islands (*Spain*); mericarps collected in the wild received from the Jardin Botánico "Viera y Clavijo" (Index Seminum 1993). Voucher specimen: S. Neves s.n. (COI).
	199	Madeira (*Portugal*), Curral das Freiras, "in convallibus et rupibus praeruptis", 12.vii.1865-1866, G. Mandon 121 (E).
	212	Canary Islands (*Spain*); mericarps collected in the wild received from the Jardin Botánico Viera y Clavijo (Index Seminum 1994).
	273	Madeira (*Portugal*), between Pico do Arieiro and Pico Ruivo, rock-ledges above path, c. 1650 m, 29.xi.1989, L. Chilton & N.J. Turland 135 (BM).
	294	Canary Islands (*Spain*), Gran Canaria, Cruz de Tejeda, between Tejeda and Roque Rublo; 1600 m, steep rocky crags, 19.vi.1995, M.F. Gardner & S.G. Knees SG 5750 (E).
B. semicompositum L.	**188**	*Portugal*: Algarve, Castro Marim, v.1887, A. Moller s.n. (E).
	189	*Spain*: Cataluña (Barcelona), Hostalets 'friches' (fallows) 24.vi.1908, F. Sennen 557 (E).
	286	*Spain*: Ciudad Real, Daimiel, Tablas de Daimiel, Isla del Morenillo, 12.v.1992, S. Cirujano (MA 552469).
B. stellatum L.	**133**	Mericarps collected from cultivated material; received from the Botanischer Garten der Johann Wolfgang Goethe Universität, Frankfurt am Main, Germany (Index Seminum 1994).
	312	*Switzerland*: Canton du Valais, Col du Simplon, 'sous la statue de l'Aigle', 2000 m, 10.viii.1988, B. de Retz 88690 (MAF 145370).
B. subspinosum Maire	**299**	*Morocco*: High Atlas, S slope of Jbel Angour, c. 10,000 feet, dry rocks, 21.vii.1976, C.J. & A.R. Humphries 99 (BM).

Taxa	Acc. No	Origin of plant material
Bupleurum tenuissimum L.	**90**	*Portugal*: Beira Litoral, Figueira da Foz, Vila Verde, near a lamp factory, 40°08' N - 8°48' W, c. 2 m, marshland, growing on dry soil, 16.ix.1994 (mericarps collected 07.xi.1994), S. Neves 22 (COI, E).
	187	*Hungary*: Budapest, Kelenföld, "in pratis salsis territ", 22.viii.1913, G. Moesz s.n. (E).
	233	The same as Acc. No 90 (leaf and fruit material collected 30.ix.1996).
Heteromorpha arborescens (Spreng.) Cham. & Schltdl.	**197**	*Tanzania*: Kilimanjaro, 1530 m, x.1893, G.L.A. Volkens 2649 (E).
H. transvaalensis Schltdl. & H.Wolff	**204**	*South Africa*: Transvaal, Potgietersrus district, Makapangsgat, 1900 m, above SE cliff, 18.iv.1950, B. Maguire M2283 (E) – stems woody, erect, spreading.
Hohenackeria exscapa (Stev.) Koso-Pol.	**194**	*Turkey*: Konya prov., Konya to Çumra, Kuçuk Koy, cultivated land, 24.v.1962, Helbaek 2510 (E) – flowers white.
Nirarathamnos asarifolius Balf.f.	**195**	Socotra island (*P.D.R. Yemen*): Shihali, c. 9 km SSE of Hadiboh, 1150 m, lichen rocky slopes, 6.iii.1989, A.G. Miller *et al.* M8679 (E) – woody-based herb to 50 cm, leaves bright green, leathery, flowers white.

Appendix III

Summary of some DNA techniques

1) DNA-DNA hybridization

- Total DNA is extracted from tissue samples, and then is fragmented using a ultrasonic cell disruptor to obtain segments of c. 500 bp long. The DNA solution is then cooled to allow pairing of repetitive DNA which have a very high number of copies throughout the genome; single-copy sequences will remain unpaired.

- Repetitive DNA is removed after the solution is passed through a column which specifically binds double-stranded DNA, leaving only single-copy sequences – this also reduces viscosity of solution.

- Part of the samples (single-copy DNA) is then radioactively labelled (duplicated in the presence of nucleotides including a radioactive isotope).

- Labelled DNA fragments ('**tracer**') of one species are reacted with a much larger sample of unlabelled DNA fragments ('**driver**') of the same species (**homoduplex** reaction) and with unlabelled DNA of another species (**heteroduplex** reaction).

- Each sample is placed in a column that binds double-stranded DNA, and then is gradually heated up to 95°C. With the increase of temperature, portions of the double-stranded DNA will dissociate into single strands which are then eluted from the column into a vial. Radioactivity of the solution in the vial is measured at each step, and a 'melting profile' (a curve that shows thermal stability) can be constructed.

Complementary strands of DNA from two different species can anneal, but there would be some base pair mismatch because of divergence of sequences. With the increase of mismatch, the DNA duplex becomes less stable, with diminution on the value of temperature where the strands will dissociate ('melting' point). Differences between homoduplex and heteroduplex curves are then used to estimate genetic distance between species (Avise, 1994, p. 53-57; and Werman *et al.*, 1996).

For a comprehensive description of this technique (protocols included) see Werman *et al.* (1996). See section 9.1.1 for brief discussion on the limitations of DNA-DNA hybridization.

2) **RFLPs** – *Restriction Fragment Length Polymorphisms*

- Total DNA (or only chloroplast DNA) is extracted from plant tissue, and is incubated at 37°C in a buffer containing one or more restriction endonucleases during 4-5 hours (time enough for complete digestion of DNA).

- Fragments of the restriction digest are separated according to size by electrophoresis on gel (agarose or polyacrylamide gels). The electrophoresis buffer contains ethidium bromide that stains double-stranded DNA which can then be visualized under UV light (see section 9.2.4); there are other methods to detect DNA fragments – see Dowling *et al.* (1996).

If total DNA (= genomic DNA) is used, the number of fragments obtained will often be so large that we can only observe a smear of fragments of many sizes with unclear bands.

The first and most commonly used method to reduce the number of bands is the *Southern hybridization* which was developed by E.M. Southern (see e.g. Brettschneider, 1998; or Griffiths *et al.*, 1996, p. 442-443):

1) DNA fragments are denaturated and transferred by Southern blotting from the gel onto a solid support (e.g. nitrocellulose filter or nylon membrane) – blotting uses capillarity, so that bands will stay in the same place when transferred.

2) The membrane with the fragments is then incubated in a solution with a labelled single-stranded DNA probe (cloned DNA). Hybridization will then occur between the probe and the fragments on the membrane with a complementary sequence. The bands can now be visualised and compared (the label of the probe has generally been a radioactive isotope; but non-radioactive labels are now available).

An alternative method is to use the polymerase chain reaction, or **PCR** (see section 9.2.5), to amplify a particular segment/ gene from the extract of total DNA, and then use restriction enzymes to digest the amplified segment (this method is sometimes abbreviated as **PCR–RFLPs**).

For comprehensive description of RFLPs methods see Dowling *et al.* (1996). See also section 9.1.1 for brief discussion on advantages and limitations of RFLPs.

3) AFLPs – *Amplified Fragment 'Length' Polymorphisms*

- Genomic DNA is digested with two different restriction enzymes (or 'cutters'), one with a recognition sequence of 4 bp ('frequent cuttter'), and the other of 6 bp ('rare cutter').

- After complete digestion, specific 12-20 bp oligonucleotides ('adapters' or 'linkers') are added to the ends of the DNA fragments. The oligonucleotide compatible with the end created by the 6 bp cutter is biotinylated (i.e. it has attached a biotinyl residue – the acyl group derived from biotin – which has high affinity to the protein streptavidin). The majority of the fragments is produced by the 4 bp cutter; therefore, the biotinylated linker will be only attached to a small part of the DNA fragments.

- The biotinylated fragments are subsequently separated by binding to beads coated with streptavidin.

- A part of the biotinylated fragments is amplified using PCR. To further reduce the possible number of PCR products (bands in the gel), the primers are designed not only to be complementary to the oligonucleotide linkers that were attached to the fragments, but also to a few additional bases, i.e. the primers extend into the restriction fragments. The number of these extra bases in the primer can be adjusted to obtain a readable pattern of bands. As in RAPDs, fragments are amplified only when primer binding sites are within a distance from each other that allows amplification.

- The last stage is the electrophoresis of PCR products in a denaturing polyacrylamide gel; fragments (bands) are detected by autoradiography (primers are radioactively labelled). The result is a pattern of bands (like a bar code) that is often designated *fingerprint* (see also 'Microsatellites and minisatellites' in section 9.1.1).

For further information on the AFLP technique see Vos *et al.* (1995) and Matthes *et al.* (1998). See section 9.1.1 for discussion on advantages and limitations of the AFLP technique.